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CROSS STRIATION ALIGNMENT IN SKELETAL MUSCLE

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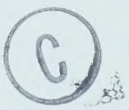

G. STUART CAMERON

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

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The undersigned certify that they have read and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Cross Striation Alignment in Skeletal Muscle" submitted by G. Stuart Cameron in partial fulfillment of the requirements for the degree of Master of Science (Surgery).

ABSTRACT

Cross striations are a distinctive feature of skeletal muscle. They represent the regular array of contractile material within muscle fibers. Hill, in 1964 while studying the extracellular fluid volume in toad sartorius muscle, noticed a tendency for alignment of cross striations between adjacent muscle fibers. That is, A band was opposite A band, I band was opposite I band, and Z line was opposite Z line, between adjacent muscle fibers. He defined, then measured, this tendency for alignment and found 49% of 2700 adjacent pairs to be aligned. He attributed alignment to be a result of longitudinal splitting of muscle fibers during fetal development.

A review of the literature suggested that muscle fibers did not develop through a process of longitudinal splitting but rather by a process of fusion of cells. This and other considerations led to a rejection of Hill's hypothesis that alignment was a result of longitudinal splitting. This study was initially concerned with confirmation and extension of Hill's observations followed by a proposal of an alternate hypothesis and testing of two of its implications.

A randomized technique of measuring alignment of cross striations between adjacent muscle fibers was developed and its accuracy tested. Alignment was measured in rat lumbrical, sartorius, soleus, gastrocnemius, tongue, and lateral rectus muscles; in frog (Xenopus laevis) sartorius, iliotibialis, and anterior tibialis muscles;

and in dog lumbrical and sartorius muscles; and was found to be present with high statistical significance.

The morphological features of striation alignment were studied in both longitudinal and oblique sections of rat lumbrical and frog sartorius muscle. The descriptions made by Hill were confirmed. It was noticed also that on oblique sections that mutual alignment often occurred in groups of fibers, and this was confirmed quantitatively.

The hypothesis proposed as an alternate to Hill's stated that alignment was a condition in which shear forces between adjacent muscle fibers were minimized during contraction. This hypothesis implied that lateral connections existed between the sarcomere and the sarcolemma, and that the sarcolemma moved with local contraction of the sarcomere. Both of these implications were tested. Rat lumbrical muscles swollen with hypotonic solution prior to fixation were examined with the electron microscope. Thin filaments and vesicles connecting the Z and M bands to the sarcolemma were found. Ballooning of the sarcolemma between these attachments was observed. Similar structures in frog sartorius muscle spindle were observed. The literature regarding lateral connections of contractile material to the sarcolemma was reviewed and the possible nature of these structures was discussed. The second implication was tested by electrically stimulating small bundles of frog sartorius muscle and observing local movement of the sarcolemma with the sarcomere. These two preliminary items of data supported the hypothesis that the condition of alignment tended to reduce shear forces between muscle fibers during contraction.

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INTRODUCTION

Alignment of cross striations between adjacent muscle fibers was first noted by Buchthal and Knappeis, in 1940, in living muscle fibers. D. K. Hill, in 1964 while studying the space in frog sartorius muscle to which albumin was accessible, described "phasing" of cross striations between fibers in very close contact. In 1965 he discussed this more thoroughly and measured the degree of alignment. Between adjacent muscle fibers sarcomere position can range from perfect alignment, with the Z, A, I, and M bands of one fiber exactly opposite their counterpart of the other, to exact misalignment with the Z band of one fiber exactly opposite the M band of the other. Hill divided this range into six equal divisions and defined position 1 to be the position of alignment; position 6, the position of misalignment; and positions 2, 3, 4, and 5 to be intermediate. In longitudinal sections of frog sartorius muscle (Bufo bufo) he measured 2700 positions of sarcomere pairs and found alignment in 1333 (49%) with progressive fall of frequency to the position of misalignment. He described runs of 30 to 40 consecutive alignments interspersed with "vernier shifts" and noted curving, branching, and faults (nonius) of cross striations within fibers with the appearance of maintaining alignment. He did not find structures between fibers with electron microscopy which would account for alignment. He proposed that following longitudinal division

of muscle fibers during the developmental stages there was incomplete separation with the cross striations maintaining their original register. In the literature there is not only little evidence that muscle fibers split longitudinally during development, there is contrary evidence such that the validity of Hill's hypothesis is doubtful. Therefore a study of alignment of striations in muscle fibers has been carried out to confirm and extend Hill's original observations on frog muscle, to investigate whether the phenomenon is general for vertebrate skeletal muscle, and to search for an explanation.

LITERATURE REVIEW

Hill proposed that cross striation alignment was due to incomplete separation following longitudinal splitting of muscle fibers during fetal development. A search of the literature on the histogenesis of muscle relevant to this hypothesis was made.

1. Definition of Cell Types in the Histogenesis of Muscle

Myogenesis begins in the early embryo and the adult complement of muscle fibers is obtained at the latest shortly after birth. The cell types were classified by Boyd in 1960. The premyoblast in the early embryo is characterized by its location and slight elongation rather than by any specific feature. The myoblast is larger and more elongated with one or several nuclei. It contains filaments and some myofibrils. The myotube is a long multinucleate cell. The nuclei are lined up longitudinally in the center of the sarcoplasm and the myofibrils occupy a peripheral position forming a tube-like appearance on cross-section. The myotube matures into a muscle fiber with peripheral migration of nuclei to lie under the sarcolemma and with growth in size and number of myofibrils.

2. Multiplication of Mononuclear Cells

Early in myogenesis, myoblasts and small myotubes are found in groups of 3 to 20 with very close apposition of cell membranes. The

cell membranes are less than 400 Å apart. There is either no basement membrane or a thin common basement membrane present between them. They interdigitate with one another with pseudopodia-like projections of the cytoplasm. Tight junctions are present. Mitotic figures are frequently seen in the mononuclear cells at this stage.

In the myotube stage mononuclear cells are abundant. MacCallum, in 1898 in the sartorius muscle of the human embryo, described central vesicular nuclei of myotubes and peripheral solid nuclei of mononuclear cells. Morpurgo, in 1898, referred to 'not yet differentiated cells' in rat muscle. Boyd, in 1960, citing Couteaux, described 'satellite cells' as uninucleated elements covering primary myotubes, multiplying by mitosis, and developing into second generation fibers. Hay, in 1961, described mesenchymal cells associated with muscle fibers in the tail of the salamander. MacConnachie et al, in 1964, described division of nuclei in muscle fibers of the postnatal rat using cholchicine to arrest division in metaphase. The dividing nuclei were partially surrounded by a thin rim of cytoplasm which appeared to have fine matrix and different staining properties than the rest of the cytoplasm. The line of demarkation between these two types of cytoplasm was usually quite sharp and on occasion there appeared to be a cleavage between them. It was their opinion that the dividing nuclei were not part of the muscle fiber but rather closely opposed satellite cells. Chikaulas and Pauly, in 1965, in studying the postnatal growth of skeletal muscle in the rat found undifferentiated myoblasts present up to three weeks of age. They tended to be more abundant at the periphery of bundles of myotubes. Pryzbylski et al,

in 1966, described numerous undifferentiated cells surrounding newly formed myotubes. These myoblasts underwent nuclear division and they fused with myotubes. Ishikawa, in 1966, in a study of adult satellite cells, described 'fibroblast-like cells' around and between myotubes in the early muscle fiber stage. These cells had dark unevenly stained nuclei, granular endoplasmic reticulum, many ribosomes, but no glycogen granules or myofibrils. Their cell membranes were closely applied to the sarcolemma of myotubes without intervening basement membrane. The membranes were only 150 Å apart. He proposed that the mononuclear satellite cell of the adult developed from the 'fibroblast-like cell' of the early muscle fiber stage. He also noticed, occasionally, satellite cells in pairs, as if they originated from one cell which had undergone mitosis.

The presence of mononuclear cells in muscle from the early embryo to the adult appears to be well established. These cells can be interpreted as myoblasts and are probably represented as satellite cells in the adult muscle. They undergo mitotic division at all stages of muscle development.

3. Multinucleation of Myotubes

The myotube has many nuclei dispersed longitudinally throughout the center of the sarcoplasm. In the course of development from the mononuclear myoblast through the myotube stage to the adult muscle fiber there is an increase from one nucleus to several hundred. This increase can come about by one of two ways. Either there is some type of division of nuclei within the multinucleated cells, or there is

division of nuclei of mononuclear cells with subsequent fusion of cells to form multinucleated cells.

Nuclear division has never been shown to occur in multinucleated myotubes or muscle fibers. A great deal of discussion in the literature in the past fifty years has been made on the occurrence of amitosis in multinucleated muscle cells where the nucleus divides without having undergone mitosis. Capers, in 1960, thoroughly review this subject and finds little to support this idea. Kelly and Zacks, in 1969, cite many recent studies which have demonstrated that neither mitosis or amitosis occur in multinucleated muscle cells.

If nuclear division occurs only in mononuclear cells and not in multinucleate cells, how then do muscle fibers obtain their numerous nuclei? Many authors have proposed that multinucleation occurs with fusion of myoblasts, beginning with Schwann, in 1839, and later Heidenhain, in 1899. Dessouky and Hibbs, in 1965, have described the electron microscope characteristics of what appeared to be myoblast fusion. There was, firstly, blebbing of the membranes between closely opposed cells, followed by discontinuities and vacuoles, and then disappearance of the membrane. Other workers have also described this appearance.

Myoblast fusion has been observed by many in tissue culture since 1957. Embryonic myogenic cells first divide mitotically. Then they fuse into multinucleated muscle fibers which subsequently form cross striations and undergo rhythmic contraction. In tissue culture mitosis can only be observed in mononucleated cells. Okazaki and Holtzer, in 1966, using chick embryo somite in culture and techniques

of autoradiography with tritiated thymidine to demonstrate DNA synthesis, colchicine to arrest nuclear division in metaphase, and fluorescent antibody labelling of myosin, found that nuclear division and DNA synthesis occurred only in mononuclear cells. They found that in cells which undergo division of nuclei there was no myosin. In those cells which did contain myosin there was no division of nuclei. Konigsberg et al, in 1960, with chick embryo muscle cultures found that DNA synthesis and mitosis of cells could be arrested with nitrogen mustard, yet multinucleated cells could still form. At the same time there was no change in the total number of nuclei and the number of mononuclear cells decreased. The converse experiment was done by Stockdate et al, in 1963. They were able to prevent fusion of mononuclear cells with 5-bromo-deoxyuridine. DNA synthesis and division of cells by mitosis still occurred. Przbylski et al, in 1966, in reviewing myoblast fusion stated that all synthetic events leading to formation of a striated muscle cell nuclei occurred in mononuclear cells and that multinucleated cells were formed by ensuing fusion. Studies of muscle regeneration by Bintliff and Walker, in 1960, and Lash et al in 1966, both with mice, were in agreement.

The electron-microscopic appearance of developing muscle also suggests that multinucleation of muscle fibers is the result of fusion of cells. Kelly and Zacks, in 1969, in their study of the myogenesis of rat intercostal muscle, gave a concise account of the stages of development of muscle fibers and it was their view that multiplication of cells occurred in mononuclear cells and that multinucleated

muscle fibers were formed by ensuing fusion.

4. Increase of Numbers of Myotubes

During the myotube stage there is a great increase in the numbers of myotubes. MacCallum, in 1898, found the number of myotubes in the human sartorius muscle increases twenty times from the 74 mm. to the 170 mm. embryo. They appeared in successive generations, developing around the periphery of the first generations. Boyd, in 1960, and Kelly and Zacks, in 1969, reviewed the subject of multiplication of myotubes. They considered two theories: longitudinal splitting of myotubes and fusion of mononuclear cells.

A process of longitudinal splitting of myotubes has been considered by many to be the method by which successive generations of myotubes were formed. The very close apposition of cells and the widely held opinion that multinucleation occurred by amitosis made this a very plausible hypothesis for light microscopists. This theory necessarily implied that nuclei in multinucleated myotubes underwent division and because mitotic figures could not be found the idea of amitotic division of nuclei was widely held. The evidence against both mitotic and amitotic nuclear division in myotubes and muscle fibers has already been discussed. Longitudinal splitting of myotubes has not been observed with the electron microscope. Kelly and Zacks, in 1969, suggested that the appearance of longitudinal splitting seen with light microscopy might be due to separation of veryly closely opposed, yet separate myotubes.

The alternate theory of multiplication of myotubes was held

by Couteaux, in 1941, and by others. Their view was that new myotubes developed from mononuclear cells. Boyd, in 1960, supported this view from his personal observations. He was misinterpreted by Hill on this point and his error led him to believe that muscle fibers multiplied by longitudinal splitting. Kelly and Zacks, in 1969, have studied the development of rat intercostal muscle with electron microscopy and exemplify many previous studies. They have observed primary generations of myotubes forming with one to several undifferentiated cells very closely opposed. As growth of the primary myotube proceeded multinucleation occurred by fusion with mononuclear cells. Successive generations of myotubes formed from undifferentiated mononuclear cells in the spaces between the myotubes of the primary generations. Mature muscle was a checkerboard intermingling of generations of myotubes. Satellite cells were undifferentiated cells remaining after muscle fiber formation.

5. Growth of Muscle Fibers

Muscle bulk increases greatly from the embryo to the adult. This is a result of two processes: increase of numbers and increase in size of muscle fibers.

MacCallum, in 1898, counted the numbers of muscle fibers in cross-sections of human sartorius muscle from the 74 mm. (crown-rump) embryo to the adult and found 6509 fibers in the 74 mm. embryo, 12,840 fibers in the 170 mm. fetus and 142,118 and 136,406 fibers in the adult. This suggested that the 170 mm. fetus (6 months) possessed close to the adult complement of muscle fibers.

Very few other studies have been made quantitatively estimating the increase of numbers of muscle fibers. Morpurgo, in 1898, stated that there was no increase in numbers of fibers after birth. Chiakulas and Pauly, in 1965, found in newborn rats that the number of fibers on cross-section of the muscle increased until three weeks of age following which there was no further increase. Bloom and Fawcett in their textbook state that there are no new fibers formed in the human fetus after it reaches 13 to 15 cm.

Numbers of muscle fibers do not increase with work hypertrophy. Van Linge, in 1962, transplanted the plantaris muscle to the calcaneum in the rat then vigorously exercised the animal. While the weight of muscle doubled and the maximum contractile force tripled, there was no increase of number of muscle fibers.

From this evidence it therefore appears that there is little or no increase in numbers of muscle fibers after the late myotube stage.

In contrast, the cross-sectional area of muscle fibers increases greatly from the myotube stage to the adult. MacCallum, in 1898, found in the human sartorius muscle that the average cross-sectional area of the muscle fibers increased from 58.82 $um.^2$ in the 74 mm. embryo to 819.6 and 793.6 $um.^2$ in the adult. Other studies agree with this.

Little is known of the mechanism and degree of increase of muscle fiber length. Goldspink, in 1964, studied mouse biceps brachii muscle, in which fibers run the whole length of the muscle. In the growth of the mouse from 10 grams to 40 grams the muscle increased its length approximately 40%. This was due almost entirely to a

corresponding increase of average sarcomere length with only slight increase of number of sarcomeres.

While the data is scant is appears that the numbers of muscle fibers in the adult is established surprisingly early in fetal life and that further growth is due to great increase in the size of fibers.

STATEMENT OF PROBLEM

Cross striation alignment between adjacent muscle fibers is probably not a consequence of longitudinal splitting of muscle fibers, as has been proposed by Hill. The process of longitudinal splitting during myogenesis has been discussed and its occurrence is extremely unlikely. Even if it did occur, it is inconceivable that cross striation alignment could be maintained during the remarkable increase in size of muscle fibers.

It is felt that alternative theories to explain cross striation are required. Hill suggested the possibility of structures connecting adjacent muscle fibers. He did not find any structures using the electron microscope and innumerable studies of muscle by many observers have not brought forth any such evidence. It can be suggested that there is synchronization of protein formation between adjacent muscle fibers during the myotube stage of myogenesis when there is very close apposition of myotubes. By this means the proteins of the myofibers would be formed so that the sarcomeres of adjacent fibers were in register. However, there is no evidence in the literature of regulation of protein synthesis between muscle fibers (Kelly and Schotland, 1972). Even if such a process existed it is almost inconceivable that the register of myofibril could be maintained during the great growth for size of muscle fibers following their formation. A fourth hypothesis is suggested in this study which proposes that cross striation alignment between adjacent muscle fibers is a condition of

minimal shear where the forces between contracting fibers are minimized and alignment naturally maintained.

This thesis was initially concerned with a confirmation of Hill's observation of alignment with morphologic description and quantitative measurement. A randomized sampling process was used to measure degree of alignment. This was not used by Hill. An attempt was made to establish the generality of striation alignment in vertebrate muscle was made. Aspects of the relation of alignment to distance between fibers, angle of section of muscle, and sarcomere length were considered. The occurrence of alignment in groups of fibers was examined. Finally, two implications of the hypothesis that alignment was a condition of minimal shear between fibers were confirmed. These were the existence of lateral attachments of the sarcolemma to the myofibrils and local movement of the sarcolemma with contraction of sarcomeres.

METHOD AND MATERIALS

1. Materials

Alignment of striation was studied in lumbrical, sartorius, gastrocnemius, soleus, lateral rectus, and tongue muscles of three Wistar rats; sartorius, anterior tibialis, and iliotibialis muscles of one frog (Xenopus Laevis); and lumbrical and sartorius muscles of one mongrel dog. The rat lumbrical and frog sartorius muscles were used for detailed study of striation alignment; the other mainly for assessment of the generality of striation alignment.

2. Histologic Methods

Small specimens of fresh muscle (2 to 10 mm.) were pinned at resting length on small pieces of cork board, fixed for two hours in glutaraldehyde and phosphate buffer at pH of 7.4, and then washed for 15 minutes three times in phosphate buffer. Staining with 2% osmium tetroxide in water mixed in equal parts with phosphate buffer was carried out for 30 minutes at room temperature. Dehydration was performed with ethyl alcohol in serial concentrations of 70%, 85%, 90%, 95%, and 98% each for ½ hour. At this stage the specimens were cut into small pieces 1 to 2 mm. in length and 0.25 to 1.0 mm. in thickness. Only the blackened peripheral areas of the larger specimens were used. The small pieces were embedded in Araldite in gelatin capsules. After hardening, the capsules were trimmed and 0.5

micron thick sections were cut with glass knives using a Porter-Blum microtome. The sections were transferred to glass slides and secured with gentle warming.

The most satisfactory stain for the sections was found to be p-phenyenediamine. A freshly prepared and filtered 1% aqueous solution was flooded onto the slides for 45 minutes and then washed off with distilled water. After drying the sections were sealed with Permount and 0.17 mm. cover slips. This stain was easy to use and consistently gave good definition of the A and Z bands. Prior to the use of this stain much unsatisfactory effort was made in using potassium permanganate. Methylene blue 1.0%; toluidine blue 1.0%; and a mixture of azure blue 1.0%, toluidein blue 1.0% and sodium borate 1.0% were additional stains tested and abandoned because of poor definition of the Z line and edges of the A band. The specimens were examined and photographed with a Zeiss photomicroscope with phase contrast objectives using Kodak Plux X pan film processed with Hyfinol and Agfa-Gavaert Kopex film processed with Diafine. Prints as needed were made with Kodabromide F_3 to F_5 paper and a Leitz photoenlarger.

Electron micrographs of rat and frog muscle were taken with a Phillips E.M. 100 Electron Microscope operated at an accelerating voltage of 60 K.V. Araldite embedded specimens were prepared as described. Thin sections (gray to straw in color) were cut with glass knives on a Porter-Blum microtome. After transfer to formvar coated copper grids the sections were stained with 5% uranyl acetate in absolute methanol then rinsed by repeated dipping in absolute methanol, 50% methanol in water, and distilled water. Further staining

was carried out with 0.2% lead citrate in water for three minutes by floating the grids inverted on drops of the reagent followed by rinsing in distilled water. Electron micrographs were taken with Kodak fine grain positive film processed with Kodak D19 developer. Prints were made on Kodabromide paper, grades F_2 to F_5.

3. Methods of Measurement of Striation Alignment

(a) Definition of striation alignment

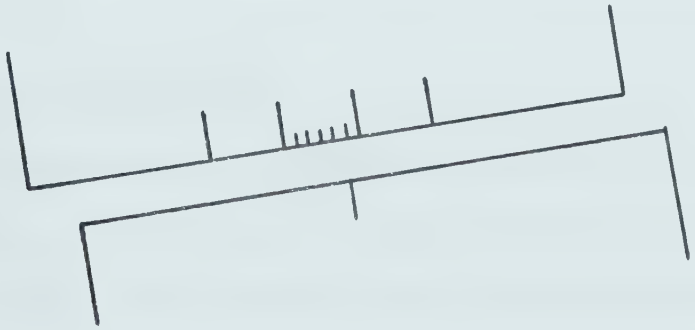
Hill's definition of striation alignment described in the introduction was used. A half sarcomere of a muscle fiber was divided into six divisions of equal length. These were numbered from 1 to 6 commencing at the division next to the Z line and ending at the division next to the M line. When the Z line of an adjacent muscle fiber lay opposite division 1, the muscle fibers were considered to take position 1, or the position of alignment. When the Z line lay opposite division 6, the fibers were considered to take position 6, or the position of misalignment. Positions 2 to 5 were intermediate.

(b) Methods of measuring sarcomere position

The important difference between this and Hill's study is that here the selection of sarcomere pairs was randomized. Photographs of muscle specimens were taken with the Zeiss photomicroscope. The film negative was placed in a photoenlarger and the image projected on a screen formed simply by a large sheet of paper with circles of 1.3 cm. diameter drawn at 10.0 cm. intervals. The area enclosed by circles was 2% of the total area of the screen. The photoenlarger was equipped with an automatic focusing device, enabling adjustment of the size of the image. Throughout the study, image size was adjusted in order that the sarcomere length was 1.2 cm. on the screen.

Following size adjustment the projected image was inspected. When some part of both fibers of a muscle fiber pair fell within one of the circles on the screen then a measurement of position was taken. Either the Z lines or edges of the A bands were used depending on which was closest to the center of the circle. These two structures usually gave sharp lines from which to measure sarcomere position.

To measure sarcomere position two small cards were used. The edge of one card was divided into three major increments of 1.2 cm. and a short segment of six 1.0 mm. divisons placed within the middle increment. The other card had one line drawn perpendicular to one edge.



Using one muscle fiber of the pair as a reference the first card was placed with the major lines lying over and parallel to the Z line or A band edge, whichever was closest to the center of the circle. The other card was placed with its single line lying over and parallel with the corresponding line of the opposite fiber. The relative position of sarcomeres was then read from the six small divisions on the reference card. With this apparatus rapid and accurate measurement of relative sarcomere positions could be made.

Occasionally the sarcomere lengths of two adjacent muscle fibers were different and by the definition of alignment stated a position from 1 to 6 could not be assigned. Therefore, when the difference in sarcomere lengths was greater than 16.7% (1/6) of the length of the shorter sarcomere, the pair under consideration was omitted. This condition was encountered almost exclusively on oblique sections and was less than 2% of cases.

One section of good quality was selected from each muscle specimen. Approximately 15 random photomicrographs were taken from the section to obtain sufficient numbers of muscle fiber pairs to count alignment. The number of individual measurements was usually in excess of 100. The frequency taken by each of the six positions was expressed in percent.

(c) Statistical methods

If there was no tendency for alignment, then one would expect each of the six positions to be taken with equal frequency; that is, 1/6 or 16 2/3%. The statistic used to estimate the significance level

with which a set of frequencies of the six positions taken differed from that of a random process was the chi square.

$$X^2 = \frac{fi - Fi}{F_i}$$

where f_i = observed frequency

F_i = expected frequency.

The number of degrees of freedom for the six possible positions was 5. The 95% significance value was 11.07 and the 99% significance level was 15.09. Chi square values greater than these indicated significant difference between the expected random distribution of position and the observed distribution of positions.

The chi square statistic was also used to compare groups of data. Contingency tables were prepared expected frequencies were calculated by:

$$F_i = \frac{R \times C}{T}$$

where F_i = expected frequency of measurements

R = row total of measurements

C = column total of measurements

T = total number of measurements for the table.

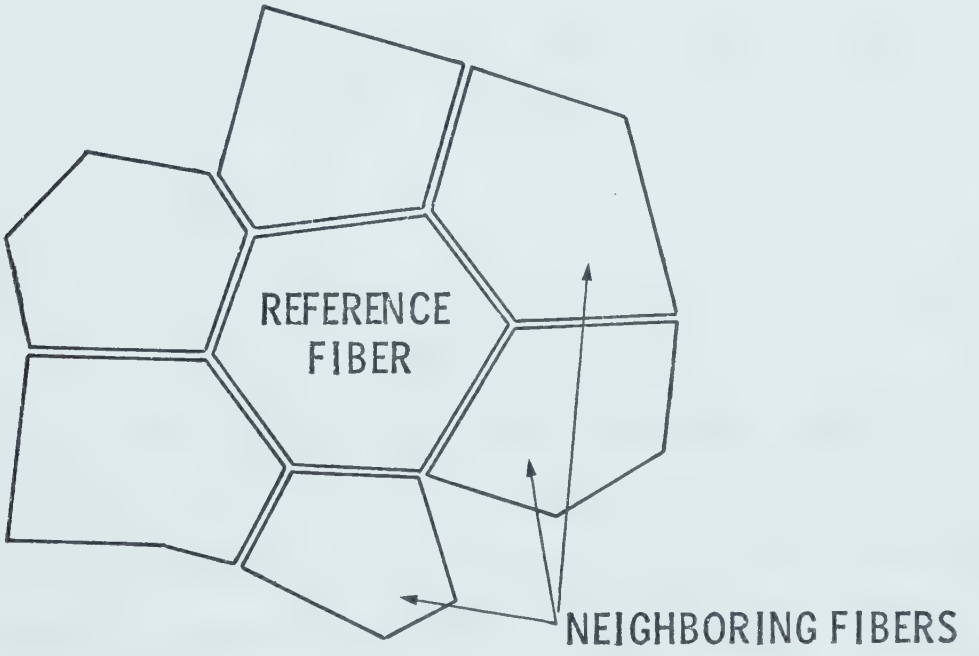
The number of degrees of freedom was calculated by

$$df = (R - 1)(C - 1).$$

Significance levels were obtained from a standard chi square table. Calculations were performed on an Olivetti-Underwood Programma 101 desk computer.

4. Methods of Measuring Groups of Aligned Fibers

On oblique sections groups of muscle fibers were often seen to be mutually aligned. The binomial distribution was used to calculate the probabilities of fibers having mutual alignment using the assumption that sarcomere positions between fibers were random and that pairs of fibers related to each other independent from other fibers. Mutual alignments were then measured and compared with expected alignment under random conditions with the chi square statistic. The binomial distribution was applied in the following manner. A group of muscle fibers was considered to consist of a single reference fiber and its immediate neighboring fibers separated by less than 1.0 microns.



In practice the number of neighboring fibers ranged between one and seven. Each was assumed to relate independently to the reference fiber.

The chance of each pair so formed being aligned was 1/6 assuming each of the six positions were taken with equal probability. The probabilities of alignments around reference fibers for numbers of neighboring fibers from 1 to 7 were calculated by the formula:

$$P_{(x)} = \frac{n!}{x!(n-x)!} p^x q^{n-x}$$

where x = number of alignments per reference fiber

$P_{(x)}$ = probability of x alignments

n = number of neighboring fibers

p = probability of alignment occurring between two muscle fibers

q = probability of misalignment occurring between two muscle fibers.

The value of p was 1/6; and q, 5/6. The recursive form of this formula was used to calculate the probabilities where:

$$P_{(0)} = q^n$$

and

$$P_{(x+1)} = \frac{n-x}{x+1} \cdot \frac{p}{q} \cdot P_{(x)} \cdot$$

For reference fibers with from one to seven neighboring fibers the probabilities of having from zero to the full complement of aligned fibers have been calculated and are shown in table 7. Reference fibers were selected from oblique pictures of rat lumbrical muscle randomly. For each reference fiber the number of neighboring fibers separated by less than 1.0 microns was counted and its sarcomere position measured. The sarcomere closest to the midpoint along the distance of apposition was used for measurement. Thus the number of neighboring fibers and number of alignments was obtained for each reference fiber. The

counted frequencies were compared with the expected frequencies from table 7 by the chi square statistic after suitable combining.

5. Other Methods

In the course of this study several procedures were used other than have been described above. For the most part these were variations of techniques which had already been used and the methods were described where appropriate.

RESULTS

1. Morphology of Striation Alignment

Typical alignment in longitudinal sections is seen in figs. 1 and 2 of rat lumbrical and frog sartorius muscle. The appearance of each of the six positions taken by sarcomere pairs can be seen frequently in all of the pictures. Long runs of alignment with "vernier shifts", extension of alignment across several muscle fibers, and curving and branching of cross striations within the muscle fibers to maintain alignment were observed. Not infrequently the register of cross striations was interrupted within the muscle fiber.

The appearance of cross striation alignment in oblique sections is seen in figs. 3 and 4 of rat lumbrical and frog sartorius. The angles of section were between 50° and 80° in reference to the longitudinal axis. Alignment extended over several muscle fibers in many areas, particularly in rat lumbrical muscle where groups of mutually aligned fibers formed a striking mosaic (fig. 3). Alignment was a feature of muscle fibers within fasciculi. With fibers separated by more than 2 to 3 microns consideration of alignment became meaningless. The sarcomere on oblique sections was seen to be a composite of several myofibrils (fig. 4) as compared to the longitudinal sections where many sarcomeres were seen along one myofibril. The curving and branching so often seen on oblique sections was due to slight changes of register between many successive myofibrils. On longitudinal sections the sarcomere length was not a composite of

many myofibrils and therefore branching and curving were not as evident. In both longitudinal and oblique sections abrupt alterations of register of cross striations (nonius) within the fiber were not infrequent. The electron microscope appearance of alignment of striations is seen in fig. 5A.

2. Quantitative Assessment of Cross Striation Alignment

(a) Accuracy of measurements

(i) Repeatability of measurements

Five successive sets of measurements (A to E) were made on rat lumbrical and frog sartorius muscles (table 1). A total of 1912 measurements were made and overall alignment was 26.2% (position 1) with high statistical significance. Variability between the five groups was low indicating that the technique of measuring sarcomere position was reliable. The chi square statistic for the five sets of measurements was 13.65, well below the 95% significance level of 43.77 for 20 degrees of freedom, indicating no difference between the sets.

(ii) Variability of measurements between two observers

One hundred and ninety-nine measurements of position on frog sartorius muscle by G.S.C. were repeated with 134 measurements by P.M. who was not aware that striations tended to be aligned (table 2). The chi square statistic was 12.44, falling between the 95% significance level (11.07) and the 99% significance level (15.09) indicating some degree of difference between the two observers. However,

both found the greater proportion of position 1 taken to be highly significant.

(iii) Sufficiency of sample size

The percentage of aligned sarcomere pairs was calculated after each of 110 measurements and plotted against number of measurements (fig. 8). Fluctuation of percentage of alignments stabilized between 42% and 48% within the first 50 measurements.

It is possible to calculate the size of sample required to estimate a mean with a specified precision provided the variance of the type of measurement is known. For example, if one assumes a standard deviation of measurement of alignment of 10% (a generous assumption), and it is desirable to state that one is 95% certain that a measurement of alignment lies within a 5% interval, then the number of measurements required may be calculated by

$$d = \frac{Z_{\frac{\alpha}{2}} \sigma}{\sqrt{n}}$$

where d = half-width of the interval, 2.5,

σ = standard deviation, 10,

$Z_{\frac{\alpha}{2}} = 1.96$,

n = sample size.

The sample size in this example is 61.

From these considerations it is evident that 100 or more measurements (desireable when using percentages) are ample to reliably express the degree of alignment for a particular muscle.

(iv) Variability of alignment within a muscle

Measurements of sarcomere position were made on three samples of frog sartorius muscle taken from proximal, middle and distal areas (table 9). Significant alignment was found in the three samples. There was no significant differences in measurements between these three groups.

(b) Survey of muscles

Significant alignment of striations was present in all muscles studied (table 3 and fig. 13). The 99.9% significance level was surpassed in all but frog tibialis anterior muscle. There was a tendency for position 2 to be taken more frequently than positions 3 to 6. The progressive decrease of frequencies from position 2 to 6 found by Hill was not observed.

(c) Effect of distance between fibers on alignment

With rat lumbrical muscle (table 4) 639 measurements of sarcomere position were made on fibers separated by less than 1.0 microns and 638 measurements were made on fibers separated by more than 1.0 microns. Alignment (position 1) was present in 44.8% of the closely opposed sarcomere pairs and in 29.0% of the widely separated sarcomere pairs. Both groups showed highly significant alignment. There was, however, greater than 99.9% significance that alignment was greater with closely opposed muscle fibers.

(d) Effect of angle section on alignment

(i) Relationship of sarcomere length to angle of section

In any muscle there is a finite number of sarcomere pairs although it may be very large. Each muscle fiber is usually adjacent to 3 to 6 other muscle fibers and each muscle fiber pair forms many sarcomere pairs depending upon the longitudinal distance of contact. When one sarcomere pair is considered the position taken will be one of the six defined positions and there should be no difference whether this is measured obliquely or longitudinally. Because alignment was seen to be a morphologic feature of both longitudinal and oblique sections, a quantitative comparison has been made.

Close examination of the sarcomeres in longitudinal sections revealed that in the lengthwise direction many sarcomere lengths were visible for each myofibril and on oblique sections each sarcomere length was composed of elements of several myofibrils (see figs. 2 and 5). To confirm that equivalent aspects of cross striations were being considered at various angles of section, the sarcomere length has been measured in sections cut from one block of rat lumbrical muscle at 10° increments from longitudinal to transverse (fig. 10). The means of between 30 and 142 measurements of sarcomere length at each angle have been plotted along with the standard deviation. The theoretical sarcomere length for each angle of section calculated on the basis of the sarcomere length of the longitudinal section has been inserted for comparison (dotted line). The measured sarcomere length corresponded closely to the theoretical length until angles of section greater than 70° were reached. The mean sarcomere length greater than 70° did nto follow the theoretical value but remained

relatively lower. It was found on examining the slides that the muscle fibers were not exactly parallel and that in transverse sections of the block most of the fibers were sectioned at a slightly oblique angle. In addition, at these high angles, small differences in angle produced wide variations in sarcomere length as indicated by the large standard deviation. However, with angles of section up to 70°, measured sarcomere lengths correlated well with the theoretical values and indicated that comparable aspects of cross striations were being considered at the various angles of section.

(ii) Comparison of alignment in longitudinal and oblique sections

In three muscles, one lumbrical each from rats II and III, and the frog sartorius, longitudinal sections were taken. Then, on the same blocks, oblique sections were taken from 55° to 75°. Measurements of alignment were made between muscle fiber pairs separated by less than 1.0 microns. There was no significant difference of alignment between the oblique and longitudinal groups (table 5). This evidence suggested that alignment between muscle fibers was comparable whether the fibers were examined longitudinally or obliquely.

(e) Effect of sarcomere length on alignment

Alignment in a rat lumbrical muscle fixed with a sarcomere length of 2.2 microns was compared to alignment in a rat lumbrical muscle fixed with a sarcomere length of 2.9 microns (table 6). No significant difference was present.

(f) Measurement of alignment occurring in groups of fibers

From oblique sections of rat lumbrical muscle 76 reference fibers were selected randomly. Both their total numbers of neighboring fibers separated by less than 1.0 microns and the numbers of neighbors with which they were aligned were counted (table 8). The numbers of reference fibers for each number of alignments were totalled. There were 18 reference fibers with no alignments, 24 with one, 27 with two, 6 with three, and 1 with four. Applying the probabilities of table 7 to these totals the expected frequencies of each class were calculated and are indicated on table 8 in brackets beside the measured frequencies. The observed frequencies and expected frequencies were compared with the chi square test. Totals for reference fibers with 2, 3 and 4 aligned neighboring fibers were combined. The totals of reference fibers for 0, 1 and the combined group of aligned neighboring fibers were 18, 24 and 34; with expected frequencies of 38, 28 and 10. The number of degrees of freedom was 2. The chi square statistic was 17.83, greater than the 99.9% significance level of 13.82. It indicated a very low probability that the degree of alignment in small groups seen on oblique sections of rat lumbrical muscle was due to chance.

To apply the binomial distribution to groups of fibers on oblique sections the null hypothesis was stated where it was assumed that there was no tendency for alignment between any fibers. It was then shown that the null hypothesis was not true for the specific case of a reference fiber and its neighboring fibers. Each neighboring fiber was considered to act independently with the reference

fiber. That is, it was assumed that adjacent neighboring fibers had no tendency for alignment. No measurement of alignment or consideration of alignment between adjacent neighboring fibers was made. However, if it is true that there is significant alignment between reference fibers and neighboring fibers, there must also be significant alignment between adjacent neighboring fibers. This means that there is a condition of mutual relationship between groups of fibers where if fiber A is aligned with fiber B and fiber B is aligned with fiber C, then fiber A must be aligned with fiber C. This consideration would tend to increase the frequency of observed alignments above that which would be expected if alignment occurred only between pairs acting as independent units. On oblique sections, striking mosaics of groups of mutually aligned fibers may be readily observed (fig. 3). The probability of this situation existing is extremely remote, considered either from random measurements of alignment of adjacent muscle fiber pairs or from applying the binomial distribution to reference and neighboring fibers on oblique sections. The situation of groups of aligned fibers can be explained on the basis of a consideration of mutual alignments. One might speculate how a simple effect, a tendency for alignment, could be translated into a morphologic pattern; but no mathematical explanation can be offered here.

The total number of pairs of muscle fibers considered in this analysis was 280 and the total number of alignments was 100, or 35.7%. This agreed well with the previous assessments of alignment in rat lumbrical muscle (table 3).

3. Hypothesis to Explain the Tendency for Alignment

Hill's hypothesis that alignment was due to incomplete separation of muscle fibers following longitudinal splitting was discussed and considered to be highly unlikely. Not only was there no evidence in the literature that longitudinal splitting of muscle fibers during myogenesis occurred, there was an implied great and remarkably ordered growth of muscle which followed. The observation that alignment occurred in groups of muscle fibers placed even more doubt on the hypothesis as it would imply several occurrences of longitudinal splitting and even greater ordering of growth.

In the presence of a definite phenomenon, a tendency for cross striation alignment, it is desirable to propose a testable explanation. It is suggested that alignment of striations in adjacent muscle fibers arises from a state of minimal shear between contracting fibers.

Shear between parallel planes is proportional to their relative velocity and inversely proportional to the distance between them.

$$T = \frac{uV}{t}$$

where T = shear

V = relative velocity

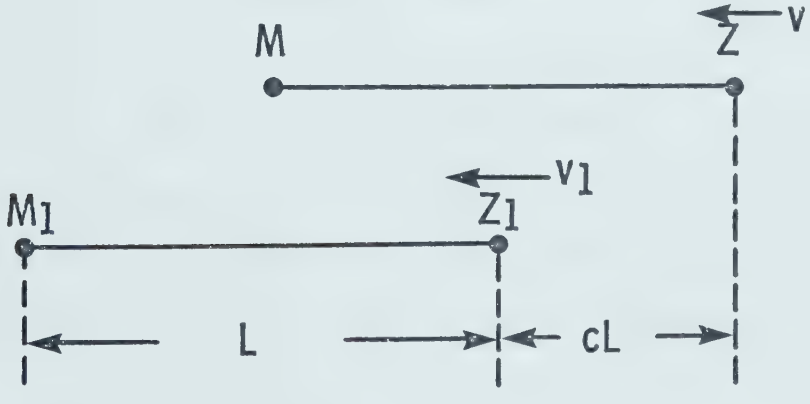
t = distance

u = constant (the coefficient of viscosity of a Newtonian fluid occupying the space between the planes).

As shear decreases and distance between the moving planes increases

one would expect the degree of alignment to decrease. This was observed in rat lumbrical muscle. This leaves only a consideration of relative velocity between muscle fibers in explaining alignment as a condition of minimal shear between muscle fibers. The proposition to be demonstrated is that the state of alignment results in minimal relative velocities between fibers during contraction.

A necessary condition for the hypothesis is that the contractile material of each sarcomere is attached to the sarcolemma in order that force may be transferred to the sarcolemma. The problem is simplified by considering half sarcomeres of two adjacent muscle fibers where myofibrils are attached to the sarcolemmas at Z and M lines. Let the two sarcolemmas be two linear (Hookian) springs, MZ and M_1Z_1, with both segments of equal length L. The segments are displaced by a distance of cL where c is a constant



which can take values from 0 to 1. The ends Z and Z_1 of the segments

move toward M and M_1 with velocities v and v_1. Since the springs MZ and M_1Z_1 are linear the velocities of points in the spring are directly proportional to their distances from M and M_1. Thus the relative velocity, V, between adjacent points in MZ and M_1Z_1 at distance X_1 and $X_1 - cL$ from M_1 and M is

$$V = \frac{v_1 x_1}{L} - \frac{v(x_1 - cL)}{L} \qquad (1)$$

rearranging

$$V = L^{-1} (v_1 x_1 - v x_1 + vcL) \qquad (2)$$

It is seen that V is minimal when vcL is minimal, that is when $c = 0$ or when there is no displacement between MZ and M_1Z_1. Taking the simplest case when $v = v_1$ then equation (2) reduces to

$$V = vc$$

Thus in this case the shearing forces between MZ and M_1Z_1 would be directly proportional to the displacement between the membrane segments. If it is accepted that shearing forces are minimal when opposed striations are aligned between muscle fibers, then it can be suggested that this position of minimal shear is attained and naturally maintained during the growth of the muscle and would represent a form of equilibrium, or of optimal position, between muscle fibers. Two important and testable assumptions have been made. The first is that sarcomere to sarcolemma attachments exist. The second is that any such attachments have sufficient strength to cause the sarcolemma to shorten with the sarcomere.

4. Preliminary Tests of the Hypothesis

(a) Lateral attachments of myofibrils to sarcolemma

Rat lumbrical muscle was immersed in phosphate buffer diluted one part with four parts distilled water for 30 minutes prior to fixation in glutaraldehyde to produce hypotonic swelling. Longitudinal sections were then examined with the electron microscope (figs. 5B and 6A-C). Myofibrils were found to be separated from each other and from the sarcolemma. Mitochondria and elements of the endosarcoplasmic reticulum were swollen and distorted. Many thin filaments were found joining the Z lines of the myofibrils to each other and to the sarcolemma. Often the filaments were replaced with small thin-walled vesicles and occasionally these occurred in short chains. Thin-walled vesicles of various sizes were frequently found near or adherent to the filaments. The filaments were connected directly to the Z lines of the myofibrils. With high magnification (fig. 6A-C), three electron micrographs are shown with filaments joining Z lines to the sarcolemma. Similar structures were also seen connecting the M lines of myofibrils to each other and to the sarcolemma but were thinner and less frequent. The sarcolemma did not appear altered by the filaments, but their presence gave the appearance of slight thickenings at lower magnifications.

A muscle spindle from the lumbrical muscle of the rat is shown in longitudinal section by electron microscopy in fig. 7A. It was fixed with a short sarcomere length. The sarcolemma was indented at each Z line and had the appearance of ballooning outward between the Z lines. Filaments joining Z and M lines to the

sarcolemma were not seen, unlike the extrafussal fibers. The sarcolemma was slightly thickened on the inner surface with a dark nubbin of material. Extending from this to the very close Z line was a slightly dense area of sarcoplasm.

A longitudinal section of muscle spindle from the sartorius muscle of the frog, Xenopus laevis, is shown in fibs. 7B and C. The muscle was fixed in a contracted state. Attachments of Z and M lines of myofibrils to each and to the sarcolemma were prominent. They had a filamentous appearance interspersed with small vesicles similar to that seen in the rat. In addition, tubular structures extended from the surface of the muscle fiber into sarcoplasm, reaching almost to the adjacent myofibril. These occurred at the level of the Z and M lines and appeared continuous with the filaments. The sarcolemma was in continuity with the tubular structures.

This evidence for lateral attachment of the myofibrils to the sarcolemma was obtained from two observations on these specimens. There was, firstly, electron dense material present between the Z and M lines of the myofibrils extending to the sarcolemma. Secondly, there was scalloping of the sarcolemma with indentations at the Z and M lines. This was most noticeable in specimens swollen with hypotonic solution prior to fixation or with specimens fixed with a short sarcomere length. The scalloping suggested that the lateral attachments possessed some degree of strength.

(b) Shortening of the sarcolemma with local contraction of sarcomeres

Shortening of the sarcolemma with local contraction of sarcomeres was demonstrated. Small bundles of frog (Xenopus laevis) sartorius muscle were isolated, immersed in choline Ringer's solution to prevent action potential propagation, and stimulated with direct current (fig. 11). Local movement of the sarcolemma was observed by placing two small silver wire riders over the bundle, separated by less than 10 sarcomere lengths. Photographs on Kodak Plus Z pan film processed with Hyfinaol were taken with a Leitz Photomicroscope using the Nomarski interference optics at 100x magnification. When the muscle was contracted, the distance between the silver wires diminished by 14%. This evidence suggested that the sarcolemma moved with the sarcomeres when they contracted rather than acting as a sleeve within which the contractile material was free to move.

DISCUSSION AND CONCLUSIONS

Morphological and quantitative data demonstrating a tendency for cross striation alignment has been presented. The hypothesis proposed by Hill to explain alignment, that it was a sequel to longitudinal splitting of myotubes during development, was discussed and discounted on three grounds. Firstly, a review of the literature suggested that longitudinal splitting of muscle fibers during development does not occur. Secondly, if it did occur, the ensuing great growth of size of muscle implied a remarkably ordered growth of muscle. Thirdly, it has been demonstrated that alignment occurs in groups of fibers, adding even greater complexity to Hill's hypothesis. Hill considered the possibility of attachments occurring between muscle fibers to account for alignment. He was unable to find any such structures with the electron microscope. This study is in agreement with Hill on this point. It has been proposed here that alignment is a state between muscle fibers in which there is minimal shear. The theoretical aspects of such an hypothesis were discussed and two testable implications were considered: sarcolemma to myofibril attachments, and movement of the sarcolemma with local contraction of the sarcomere.

Direct lateral attachments of myofibrils to the sarcolemma, particularly at the Z line, has been discounted by most authors. Wall, in 1960, stated that there was overwhelming evidence that the

Z line did not interconnect myofibrils and did not extend to the sarcolemma. He cited observations of ice crystals, nuclei, retraction bands, and nematodes freely moving up and down within the fiber. Barer in 1947 found that sarcoplasm and sarcolemma of living muscle fibers could be separated under certain conditions using dilute acid and alkaline solutions. In the electron microscope it can be seen that the cross striations of the myofibril do not extend to the sarcolemma, at least not in the same state in which they are present in the myofibril. However, these observations do not rule out tenuous direct attachments or intermittent attachments where myofibrils are connected to the sarcolemma at certain points.

The transverse tubules may be a means by which contractile force of the myofibrils is transmitted laterally to the sarcolemma. Peachy, in 1965, described transverse tubules near the Z line in frog sartorius muscle extending across the entire muscle fiber. They probably attached to the sarcolemma, with widening of the lumen and involutions of the walls at the site of attachment. Their evidence of continuity of the lumen with the extracellular space was not as definite as described by other authors. The lateral attachments from the myofibrils to the sarcolemma at the level of the Z and M lines observed in frog sartorius muscle spindle (fig. 7B) resemble this description of transverse tubules. Both Huxley and Page, in 1964, and Endo, in 1964, found that the transverse tubules were open to the extracellular fluid as demonstrated by ferritin granules and fluorescent dye respectively. Transverse tubules attach to the sarcolemma and are open to the extracellular fluid space in the

guppy (Franzini-Armstrong in 1964) and the chick (Ezerman and Ishikawa in 1967). In the mammal the evidence of direct attachment and continuity with the extracellular space is not so definite. Rather than being near the Z line the transverse tubules are usually near the A-I junction. With close attachment of transverse tubules and sarcoplasmic reticulum at the triads as well as extensive intermeshing of myofibrils and sarcoplasmic reticulum it is conceivable that forces of contraction could be transmitted in this way to the sarcolemma.

Attachment of sarcoplasmic reticulum to the sarcolemma has been previously described. In their description of sarcoplasmic reticulum, Porter and Palade in 1957 state, "Connections between the sarcoplasmic reticulum and the sarcolemma often appear in electron micrographs as irregular rows of elongated or spheroidal vesicular profiles extending transversely from a region of the reticulum to the sarcolemma at the level of the Z band. Frequent caveolae and subsarcolemmal vesicles characterized the sarcolemma at such sites". In rat cardiac muscle they describe scalloping of the sarcolemma with indentations at the level of the Z line. The sarcoplasmic reticulum beneath formed two lobe-like expansions applying themselves to the inner surface of the sarcolemma. This phenomenon has also been observed in this laboratory in rat cardiac muscle. The lateral attachments of myofibrils to sarcolemmas at the Z and M lines observed in rat muscle (fig. 5, 6 and 7A) are possibly elements of sarcoplasmic reticulum.

Lateral attachments of Z lines between myofibrils have been described by several workers. Walter et al, in 1968, found the Z and M lines in young rats encircled by sarcoplasmic reticulum and attached many numerous bands. Bennett and Porter, in 1957, found the same at Z lines in breast muscle of the fowl; Allen and Pepe, in 1965, in early chick embryos; and Bergman, in 1962, in the rat. In their study of development of the sarcoplasmic reticulum and T system in the chick, Ezerman and Ishikawa, in 1967, suggested that these structures played a role in positioning myofibrils in transverse register. Lateral attachments of Z and M lines between myofibrils were observed in this study in rat lumbrical muscle (fig. 5).

Lateral attachment of Z line to sarcolemma also occurs in cardiac muscle in a different form, the intercalated disc. While not directly pertinent to this study the intercalated discs do represent an instance where contractile material is directly connected to the sarcolemma laterally.

To support the thesis that striation alignment is a condition of minimal shear between closely opposed muscle fibers it is not only necessary to demonstrate lateral attachments of myofibrils to the sarcolemma, but it is necessary to demonstrate that they possess sufficient strength to move the sarcolemma with movement of the myofibril. The scalloping of the sarcolemma in the frog and rat skeletal muscle observed in this study (fig. 7) suggested that the structures attaching the myofibrils to the sarcolemma possessed some degree of strength. Observation of movement of the sarcolemma with contraction of the sarcomere, already described in this study,

further supports the implication that lateral attachments transmit force from the myofibrils to the sarcolemma. This observation was supported by those of Barer, in 1947, and by many observations on isolated muscle spindle in this laboratory. Huxley and Straub, in 1958, locally stimulated parts of the sarcomere with small stimulating electrodes. From their photographs and motion pictures it appeared that the sarcolemma moved locally with the sarcomere.

In this study the phenomenon of cross striation alignment between muscle fibers was examined. Four hypotheses were considered. Hill's proposal, an embryonic theory where alignment was a consequence of longitudinal splitting of muscle fibers during myogenesis, was felt to be unlikely because a literature review of myogenesis suggested that muscle fibers formed by a process of fusion of generations of mononucleated myoblasts to form myotubes, which then enlarged greatly to form adult muscle fibers. But, there is possibly a valid and very significant implication in Hill's embryonic theory. Although yet unknown, it is reasonable to suggest that alignment is present in embryonic muscle and that whatever factors are involved in the establishment of alignment are continuously present and maintain alignment during the very great growth of muscle fibers to the adult size. The remaining three hypotheses contain this implication. Hill proposed that there were anatomic connections between muscle fibers. As previously discussed, no such structures have been found. A third theory proposed was that there was some sort of chemical interreaction between muscle fibers, whereby contractile material was laid down in register. Again, there is no direct or indirect evidence to support this theory. A fourth theory, favored

in this study, is that alignment is a state of minimal shear between contracting muscle fibers. This theory is consistent with the current concepts of myogenesis and with Hill's suggestion that alignment is a feature of embryonic muscle. By this theory alignment would be established between embryonic muscle fibers when they begin to contract, then would be naturally maintained during growth. To directly test this theory it would be necessary to measure the physical forces between contracting sarcomeres: a near impossible task. However, two indirect implications were tested: the existence of attachments between the sarcomere and sarcolemma, and movement of the sarcolemma with local contraction of the sarcomere. It is felt that these two tests support the thesis that a tendency for cross striation alignment in skeletal muscle is due to a condition of minimal shear between contracting muscle fibers.

TABLE 1.--Repeatability of Measurements of Sarcomere Position

Set of measurements	Position						Total	X^2
	1	2	3	4	5	6		
A	119	51	75	60	62	79	446	39.27
%	26.7	11.4	16.8	13.5	13.9	17.7		
B	101	65	59	54	68	58	405	21.83
%	24.9	16.0	14.5	13.3	16.8	14.3		
C	119	60	67	63	64	66	439	34.86
%	27.1	13.7	15.3	14.4	14.6	15.0		
D	105	60	67	48	53	61	394	31.60
%	26.7	15.2	17.0	12.2	13.5	15.5		
E	56	25	42	36	37	32	228	14.47
%	24.6	11.0	18.4	15.8	16.2	14.0		
Total	500	261	310	261	284	296	1912	129.68
%	26.2	13.7	16.2	13.7	14.9	15.5		

X^2 for table (comparing sets of measurements A to E): 13.65.
95% significance level: 43.77 (df = 20).
There was no significant differences between the sets of measurements A to E.
X^2 for each row exceeded the 99% significance level (15.09) in all but set E indicating significant alignment.
Each set of measurements includes totals from two rat lumbrical muscles and frog sartorius muscle.

TABLE 2.--Variability of Measurements between Two Observers

Observer	Position						Total	X^2
	1	2	3	4	5	6		
GSC	62	23	29	28	26	31	199	31.20
%	31.2	11.6	14.6	14.1	13.1	15.6		
PM	44	29	21	19	13	8	134	36.69
%	33.8	21.6	15.7	14.2	9.7	6.0		
Total	106	52	50	47	39	39	333	

X^2 for table comparing the two observers: 12.44, 95% significance level: 11.07 (df = 5). 99% significance level: 15.09 (df = 5). The chance that the measurements made by the two observers differed was between 0.95 and 0.99.
X^2 for each row exceeded the 99% significance level (15.09) by a wide margin indicating that both observers found significant alignment of striations.

TABLE 3.--Survey of Muscles

Muscle	Position						Total	X^2
	1	2	3	4	5	6		
Rat lumbrical	703	342	350	279	308	266	2248	360.0
%	31.3	15.2	15.6	12.4	13.7	11.8		
Rat sartorius	74	46	27	29	8	23	207	75.75
%	35.7	22.2	13.0	14.0	3.9	11.1		
Rat gastrocnemius	74	25	30	32	23	10	194	73.65
%	38.1	12.9	15.5	16.5	11.9	5.2		
Rat soleus	92	42	21	30	37	56	278	68.72
%	33.1	15.1	7.6	10.8	13.3	20.1		
Rat tongue	32	13	17	10	6	11	89	28.24
%	36.0	14.6	19.1	11.2	6.7	12.4		
Rat lateral rectus	22	10	13	3	6	2	56	29.93
%	39.3	17.9	23.2	5.4	10.7	3.7		
Dog lumbrical	65	40	17	28	18	24	192	51.69
%	33.9	20.8	8.9	14.6	9.4	12.5		
Dog sartorius	55	26	13	21	18	12	145	52.75
%	37.9	17.9	9.0	14.5	12.4	8.3		
Frog ilio-tibialis	61	31	24	27	16	34	193	37.02
%	31.6	16.1	12.4	14.0	8.3	12.6		
Frog sartorius	240	103	115	114	101	139	812	103.9
%	29.6	12.7	14.2	14.0	12.4	17.1		
Frog tibialis anterior	26	18	17	23	9	12	105	11.74
%	24.8	17.1	16.2	21.9	8.6	11.4		

95% significance level: 11.07 (df = 5).
99% significance level: 15.09 (df = 5).

TABLE 4.--Effect of Distance between Fibers on Alignment

Distance between fibers	Position						Total	X^2
	1	2	3	4	5	6		
Less than 1.0 microns	286	101	75	61	68	48	639	377.6
%	44.8	15.8	11.7	9.5	10.6	7.5		
More than 1.0 microns	185	141	106	65	68	73	638	109.8
%	29.0	22.1	16.6	10.2	10.7	11.4		
Total	471	242	181	126	136	121	1277	

X^2 for table: 38.87.
99% significance level: 15.09 (df = 5). There was a significant difference between the two groups.

TABLE 5.--Comparison of Alignment in Longitudinal and Oblique Sections

Angle of section	Position						Total	X^2
	1	2	3	4	5	6		
Longitudinal	286	127	97	101	98	119	828	195.9
%	34.5	15.4	11.7	12.2	11.8	14.4		
Oblique	277	102	109	88	85	90	751	224.4
%	36.9	13.6	14.5	11.7	11.3	12.0		
Total	563	229	206	189	183	209		

X^2 for table: 5.67. 95% significance range: 1.145 to 11.07 (df = 5). There was no significant difference between the measurements of alignment made on longitudinal or oblique sections.

TABLE 6.--Effect of Sarcomere Length on Alignment

Sarcomere length	Position						Total	X^2
	1	2	3	4	5	6		
2.9 microns	43	14	16	10	11	11	105	46.0
%	41.0	13.3	15.2	9.5	10.5	10.5		
2.2 microns	42	16	12	17	7	9	103	47.5
%	40.8	15.5	11.7	16.5	6.8	9.6		
Total	85	30	28	27	18	20	208	90.9

X^2 for table: 3.60. 95% significance range: 1.145 to 11.07 (df = 5). There was no significant difference between the measurements of alignment made on muscle fiscal with a sarcomere length of 2.2 microns and 2.9 microns.

TABLE 7.--Probability of a Reference Fiber Being Aligned with Neighboring Fibers

Numbers of neighboring fibers	Numbers of Alignments							
	0	1	2	3	4	5	6	7
1	0.833	0.167						
2	0.694	0.278	0.028					
3	0.579	0.347	0.069	0.005				
4	0.482	0.386	0.116	0.015	0.001			
5	0.402	0.402	0.161	0.032	0.003	0.000		
6	0.335	0.402	0.201	0.054	0.008	0.001	0.000	
7	0.279	0.391	0.234	0.078	0.016	0.002	0.001	0.000

The sum of the probabilities on each of the rows is 1.0.

TABLE 8.--Alignments of Reference Fibers with Neighboring Fibers

Numbers of neighboring fibers	Numbers of Alignments				
	0	1	2	3	4
1	$1_{(1)}$	$0_{(0)}$			
2	$2_{(6)}$	$4_{(3)}$	$3_{(0)}$		
3	$9_{(12)}$	$7_{(7)}$	$5_{(2)}$		
4	$3_{(13)}$	$11_{(11)}$	$12_{(3)}$	$2_{(1)}$	
5	$3_{(6)}$	$2_{(6)}$	$7_{(3)}$	$3_{(1)}$	$1_{(0)}$
6	$0_{(0)}$	$0_{(1)}$	$0_{(0)}$	$1_{(0)}$	
Total numbers of reference fibers	$18_{(38)}$	$24_{(28)}$	$27_{(8)}$	$6_{(2)}$	$1_{(0)}$

Each figure on the table represents the number of reference fibers counted.
The numbers in brackets are expected frequencies calculated from the row totals and the probabilities on Table 7.

TABLE 9.--Variability of Alignment within a Muscle

Area of sample	Position							X^2
	1	2	3	4	5	6	Total	
Proximal	99	47	49	48	38	63	344	41.95
%	28.8	13.7	14.2	12.0	11.1	18.3		
Middle	90	35	44	46	40	47	302	39.44
%	29.8	11.3	14.6	15.2	13.2	15.6		
Distal	51	21	22	20	23	29	166	25.42
%	30.7	12.7	13.3	12.1	13.9	17.5		
Total	240	103	115	114	101	139	812	103.9
%	29.6	12.6	14.2	14.0	12.4	17.1		

The three samples (proximal, middle, and distal) were taken from one frog sartorius muscle.
X^2 for table: 3.30, 99% significance range (df=10): 2.56 - 29.59.

Fig. 1. Longitudinal Sections of Rat Lumbrical Muscle

Pictures A to D show typical cross striation alignment between muscle fibers. A and B each show alignment extending across four fibers. D is a montage of three pictures of a longitudinal section of rat lumbrical muscle showing two "vernier shifts". Relatively rapid shifts in position over several sarcomere lengths occur between runs of alignment. A sarcomere of one of the fiber pairs is dropped over each shift.

Artifact is precipitate from potassium permanganate stain. Scale bars represent 10 microns.

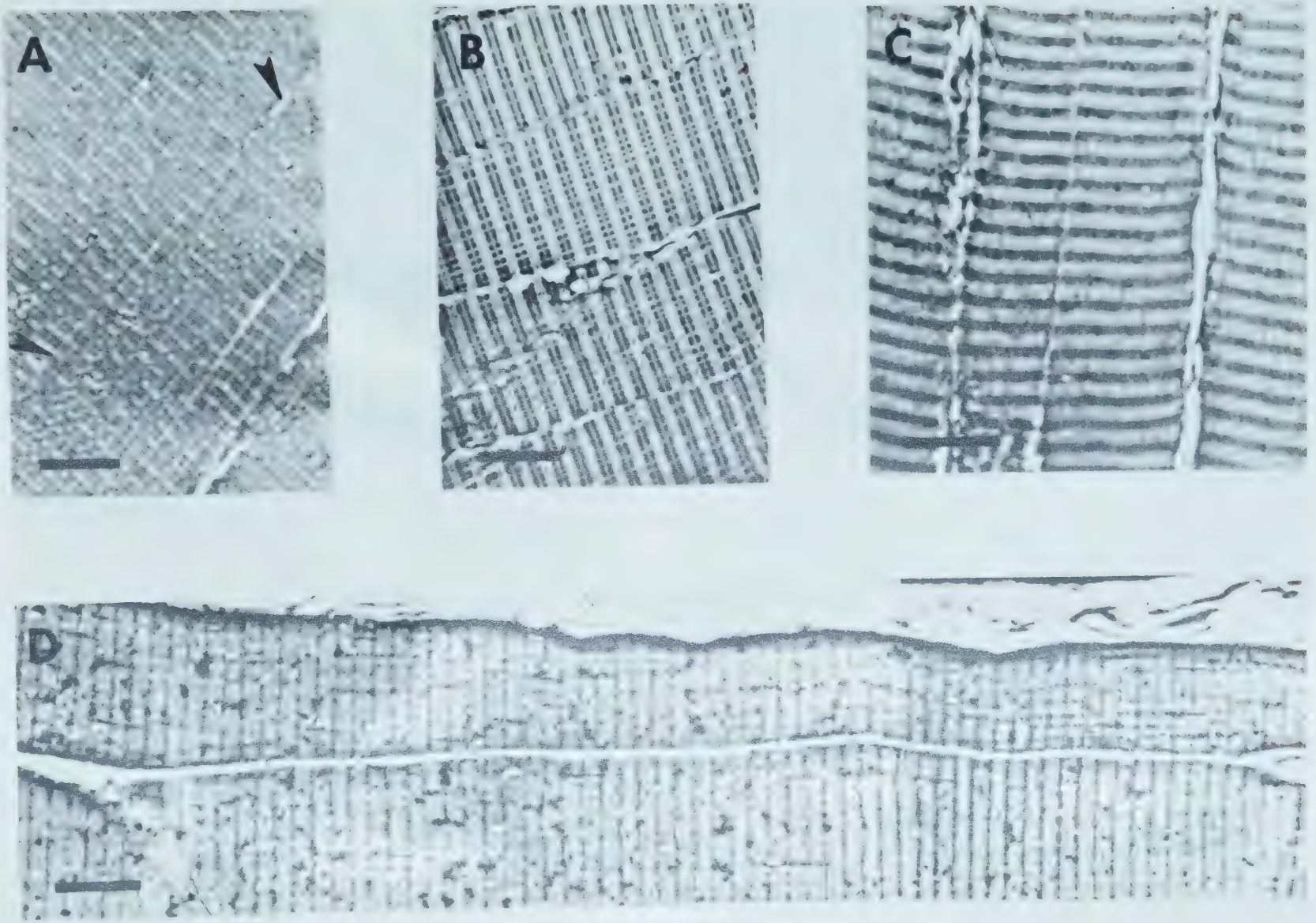
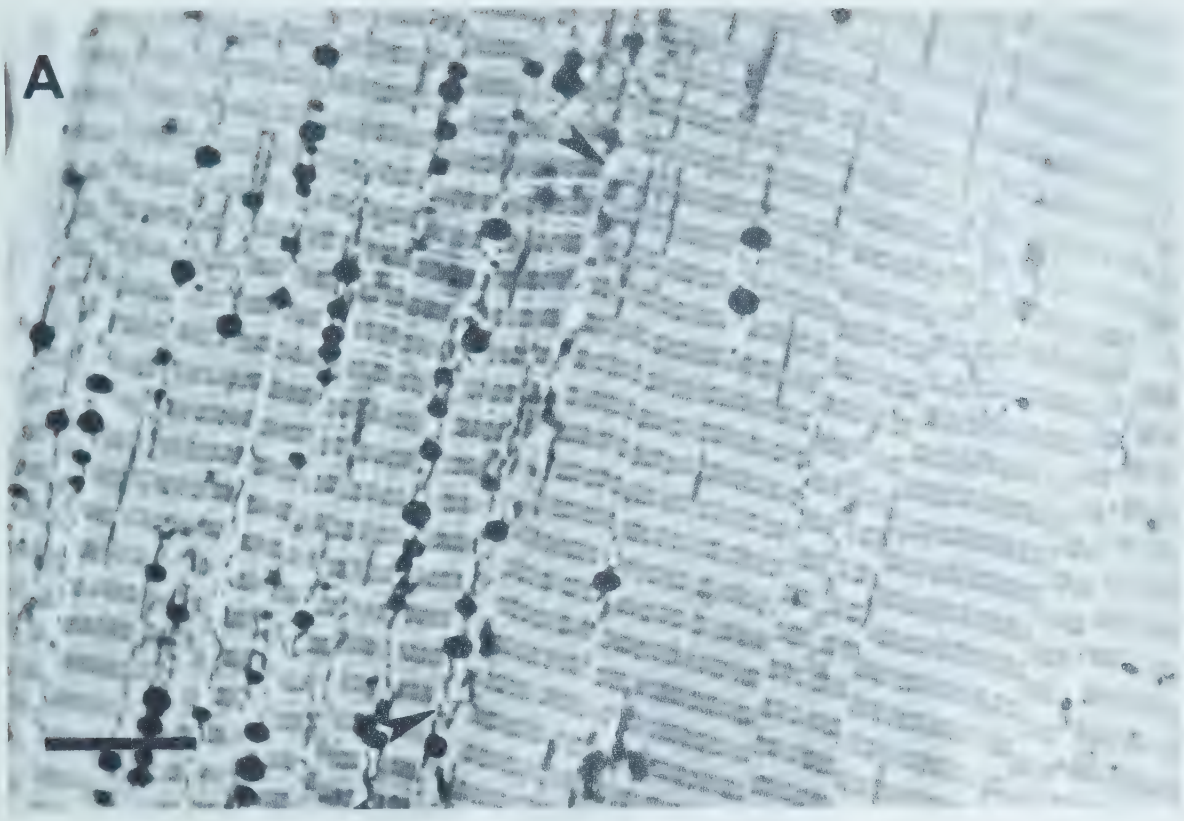

A
B
C
D

Fig. 2. Longitudinal Sections of Frog Sartorius Muscle

Typical alignment between muscle fibers is shown in frog sartorius muscle. Arrows point to the muscle fiber interspace. A "vernier shift" is present in A and cross striations appear to curve to maintain alignment. Abrupt shifts of register of cross striations (nonius) within the muscle fibers are present in both figures. p-Phenylenediamine stain. Scale bars represent 10 microns.


A

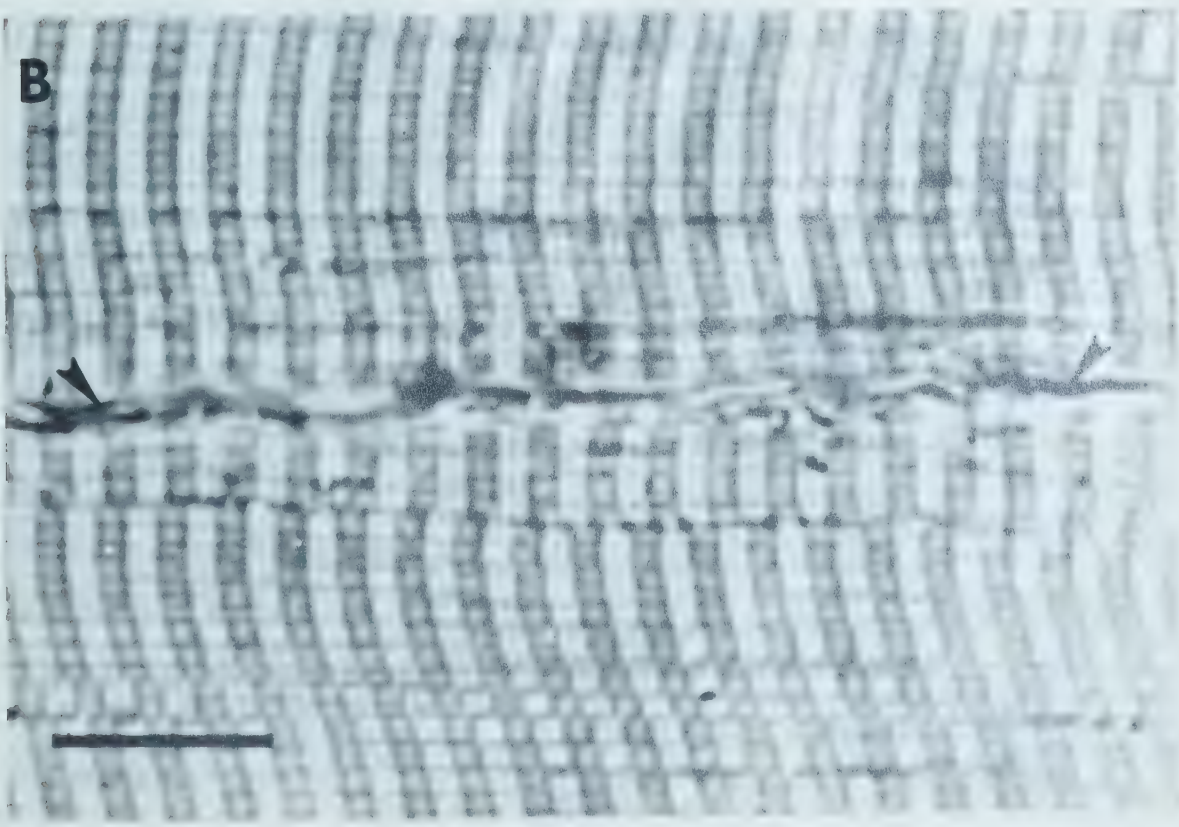

B

Fig. 3. Oblique Sections of Rat Lumbrical Muscle

These oblique sections of rat lumbrical muscle show striation alignment occurring within fasciculi. In D, mutual alignment is seen between six muscle fibers. The probability of this arrangement occurring under random conditions is less than 0.001. Potassium permanganate stain. Scale bar represents 10 microns.

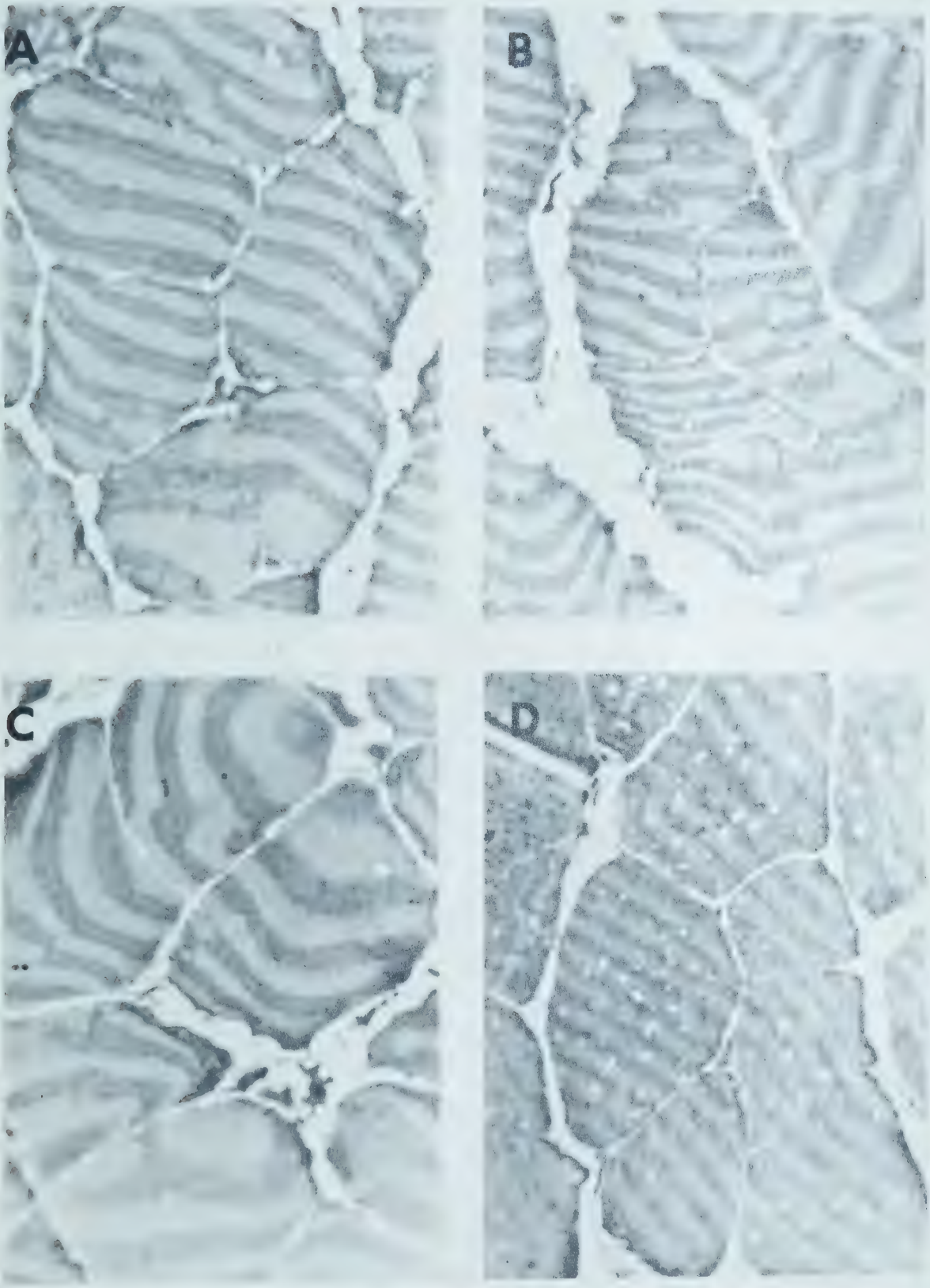

A
B
C
D

Fig. 4. Oblique Sections of Frog Sartorius Muscle

These pictures illustrate alignment between muscle fibers on oblique sections. "Vernier shifts" are present in A and B. Curving and branching of cross striations are present between adjacent fibers in C and D. p-Phenylenediamine stain. Scale bars represent 10 microns.

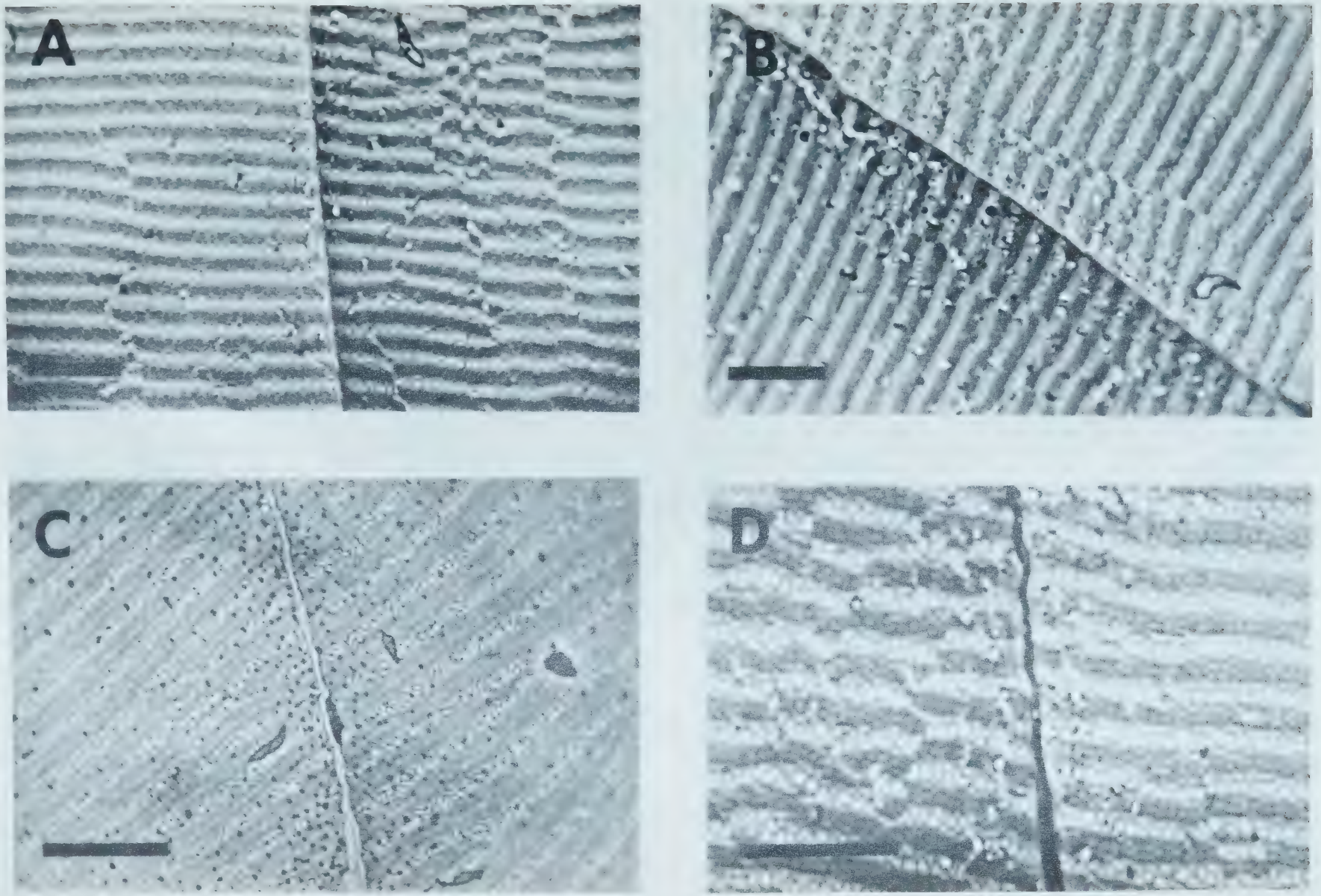
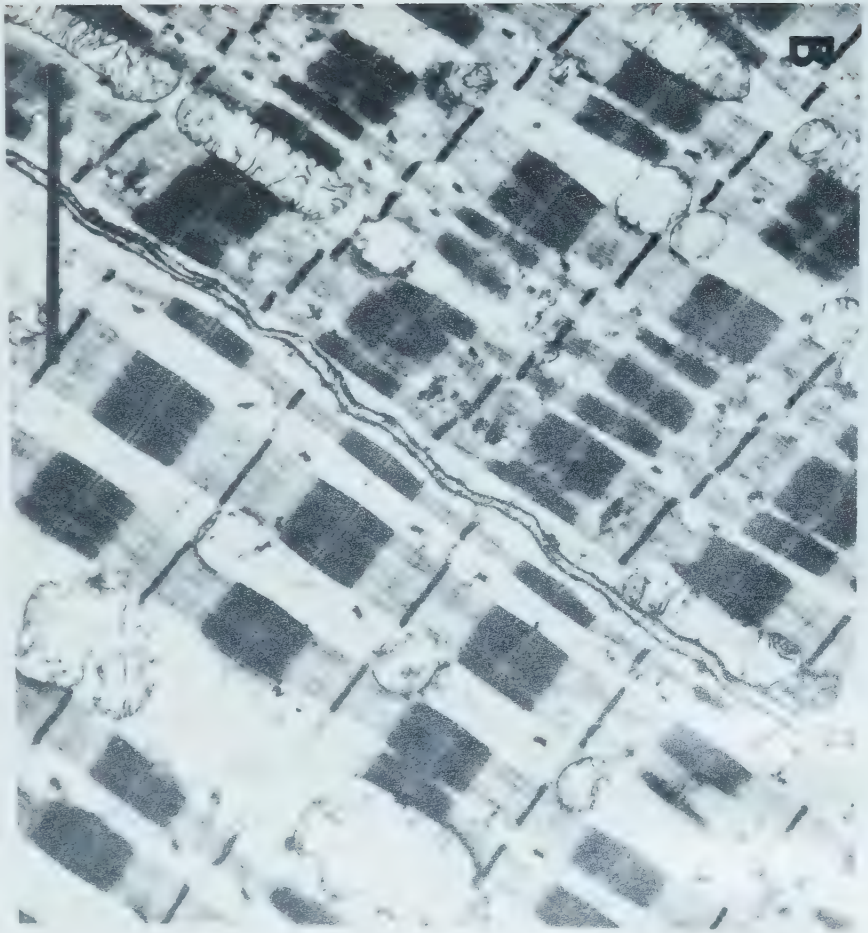

A
B
C
D

Fig. 5. Electron Micrographs of Rat Lumbrical Muscle

A is an electron micrograph of rat lumbrical muscle sectioned at a slightly oblique angle. Two muscle fibers are shown separated by approximately 0.2 microns. Sarcomere alignment was present with curving of striations in the lower fiber. This phenomenon is seen to be due to slight successive longitudinal shifts in register between myofibrils. No obvious structural attachments between the fibers were present which would account for the alignment. Scale bar represents 5.0 microns.

B is an electron micrograph of rat lumbrical muscle immersed in hypotonic solution prior to fixation. The myofibrils were separated and mitochondria and sarcoplasmic reticulum were swollen and distorted. Fine filaments or chains of small vesicles interconnected Z bands with each other and with the sarcolemma. To a lesser extent similar structures occurred at the M bands. Scale bar represents 5.0 microns.


B

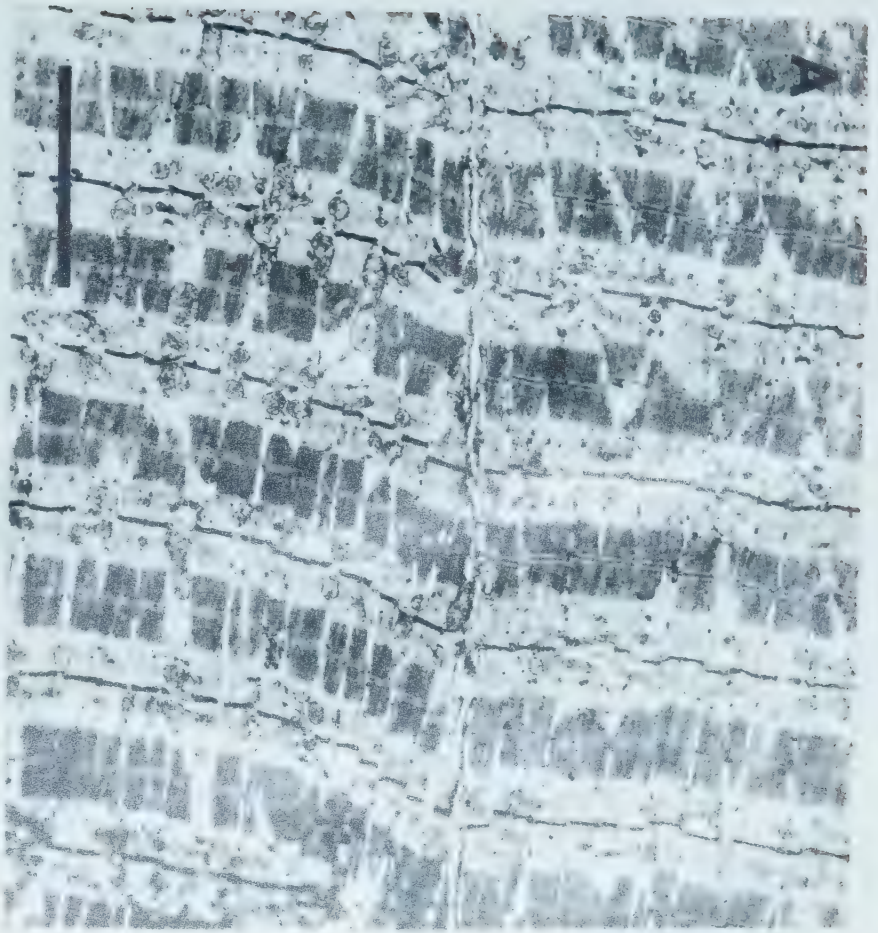
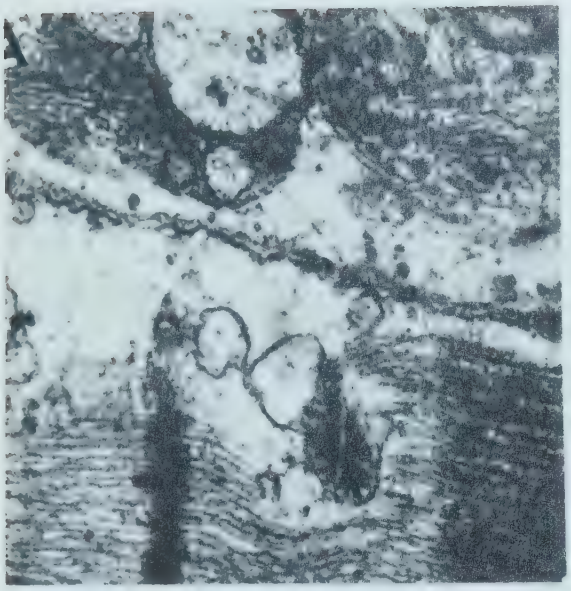
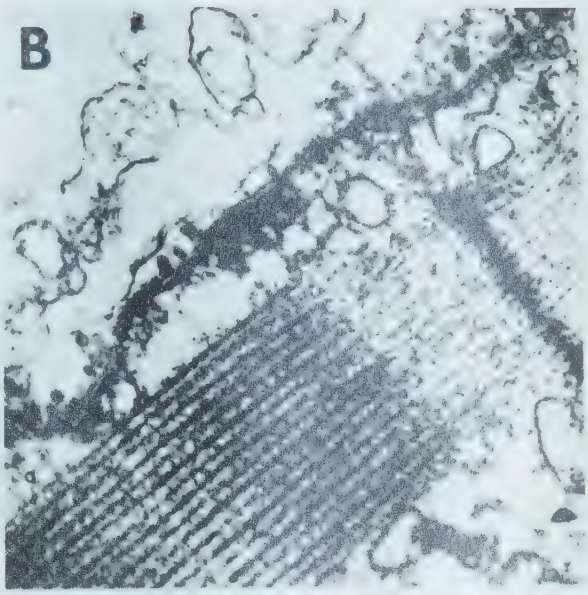

A

Fig. 6. Electron Micrographs of Rat Lumbrical Muscle

Electron micrographs taken with high magnification of rat lumbrical muscle immersed in hypotonic solution prior to fixation are shown. The myofibrils have been separated from each other and from the sarcolemma exposing the material between. Connecting structures between Z lines and sarcolemma are present. In B a similar structure is present between the M band and the sarcolemma. Scale bar represents 1.0 microns.




B

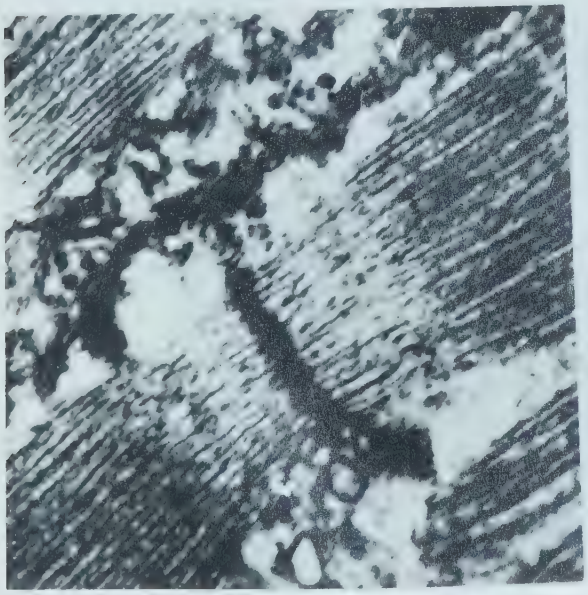


Fig. 7. Electron Micrographs of Rat Lumbrical Muscle Spindle and Frog Sartorius Muscle Spindle

A is an electron micrograph of a longitudinal section of a rat lumbrical muscle spindle fixed with a short sarcomere length. Ballooning of the sarcolemma between Z bands was present. The Z bands appeared connected to the sarcolemma with electron dense material in the form of strands and vesicles.

B is an electron micrograph of frog sartorius muscle spindle (Xenopus laevis) fixed with short sarcomere length. Structures connecting myofibrils with each other and the sarcolemma were prominent at the M and Z bands with tubular extensions from the surface of the fiber into the interior. Scale bars represents 1.0 microns.

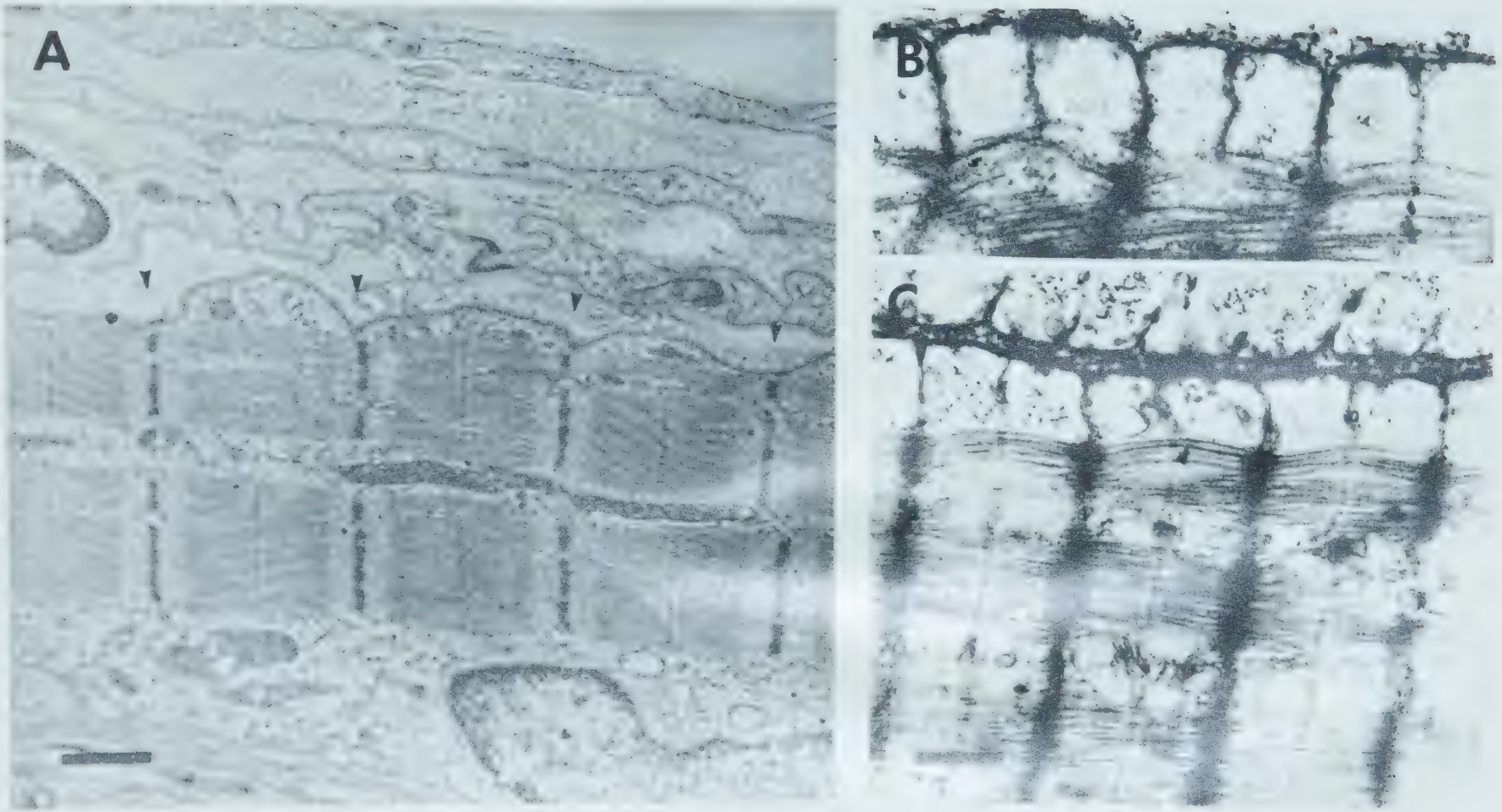

A
B
C

Fig. 8. Sufficiency of Sample Size

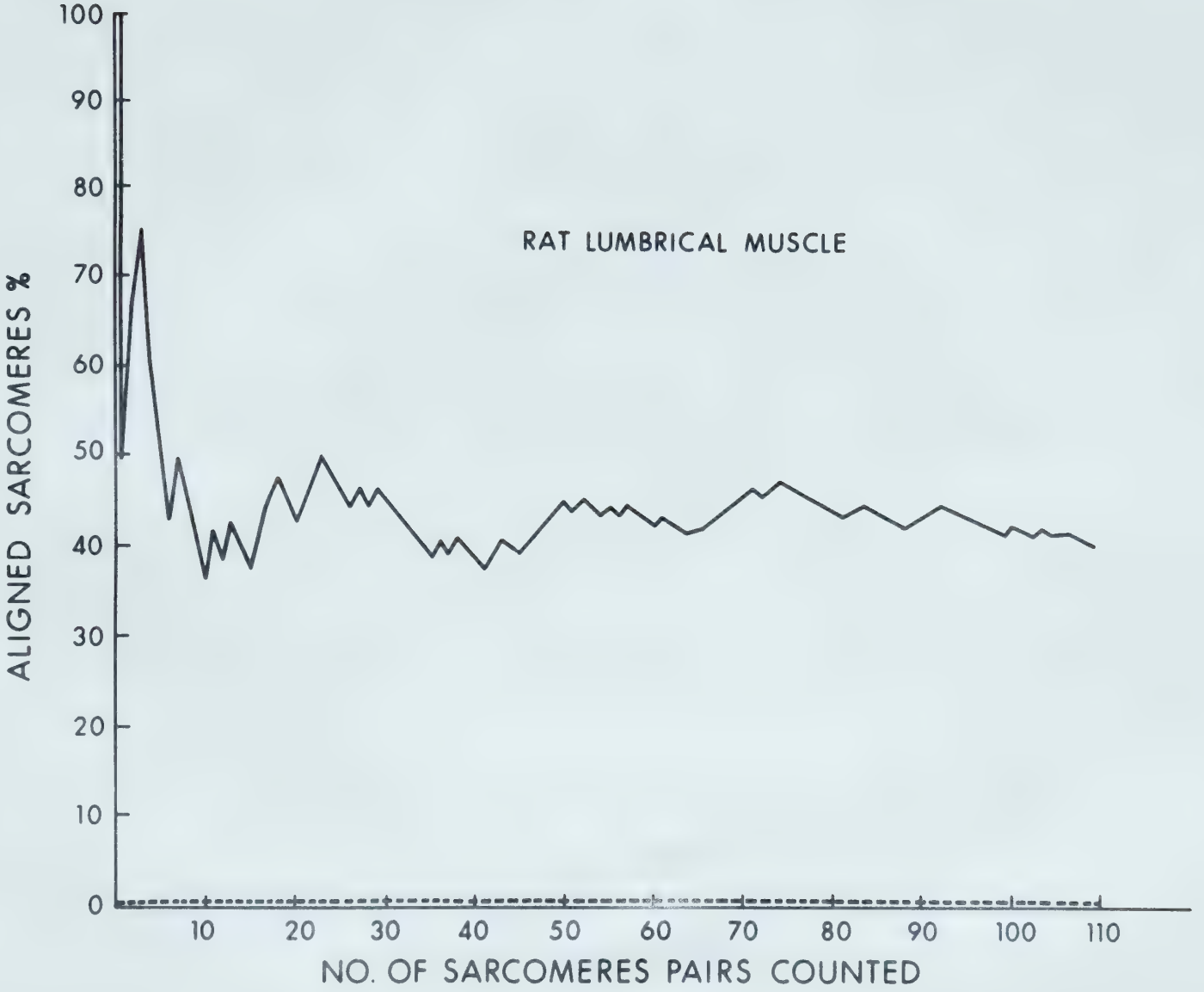

RAT LUMBRICAL MUSCLE
ALIGNED SARCOMERES %
100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
90
100
110
NO. OF SARCOMERES PAIRS COUNTED

Fig. 9. Survey of Muscles

Alignment (percent of sarcomere pairs taking position 1) is graphically illustrated for all of the muscles studied.

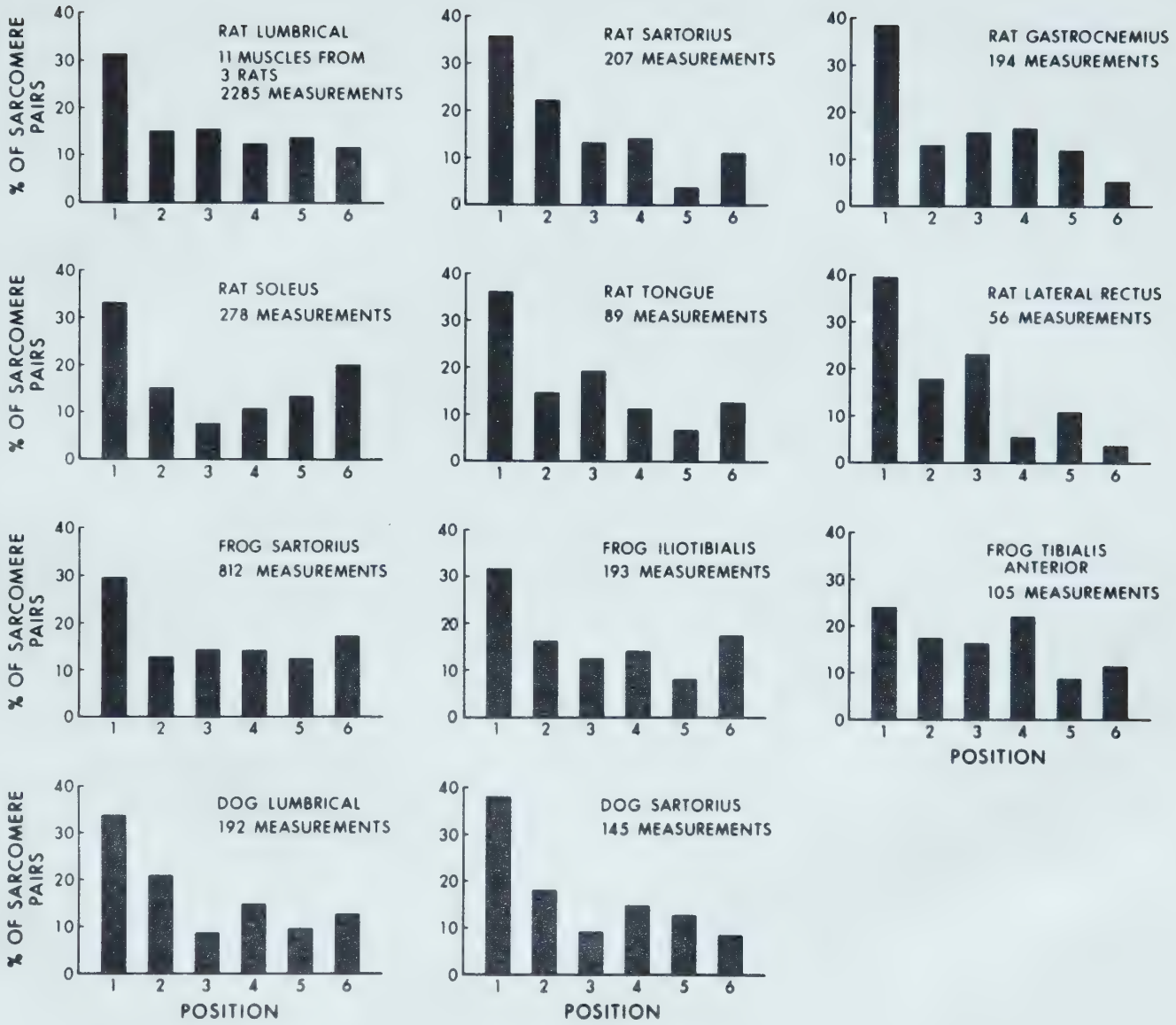

% OF SARCOMERE PAIRS
RAT LUMBRICAL
11 MUSCLES FROM 3 RATS
2285 MEASUREMENTS
RAT SARTORIUS
207 MEASUREMENTS
RAT GASTROCNEMIUS
194 MEASUREMENTS
RAT SOLEUS
278 MEASUREMENTS
RAT TONGUE
89 MEASUREMENTS
RAT LATERAL RECTUS
56 MEASUREMENTS
FROG SARTORIUS
812 MEASUREMENTS
FROG ILIOTIBIALIS
193 MEASUREMENTS
FROG TIBIALIS ANTERIOR
105 MEASUREMENTS
DOG LUMBRICAL
192 MEASUREMENTS
DOG SARTORIUS
145 MEASUREMENTS
POSITION

Fig. 10 Sarcomere Length Compared with Angle of Section

The dotted line represents theoretical sarcomere length. The solid line indicated measured sarcomere length. The vertical lines are standard deviations.

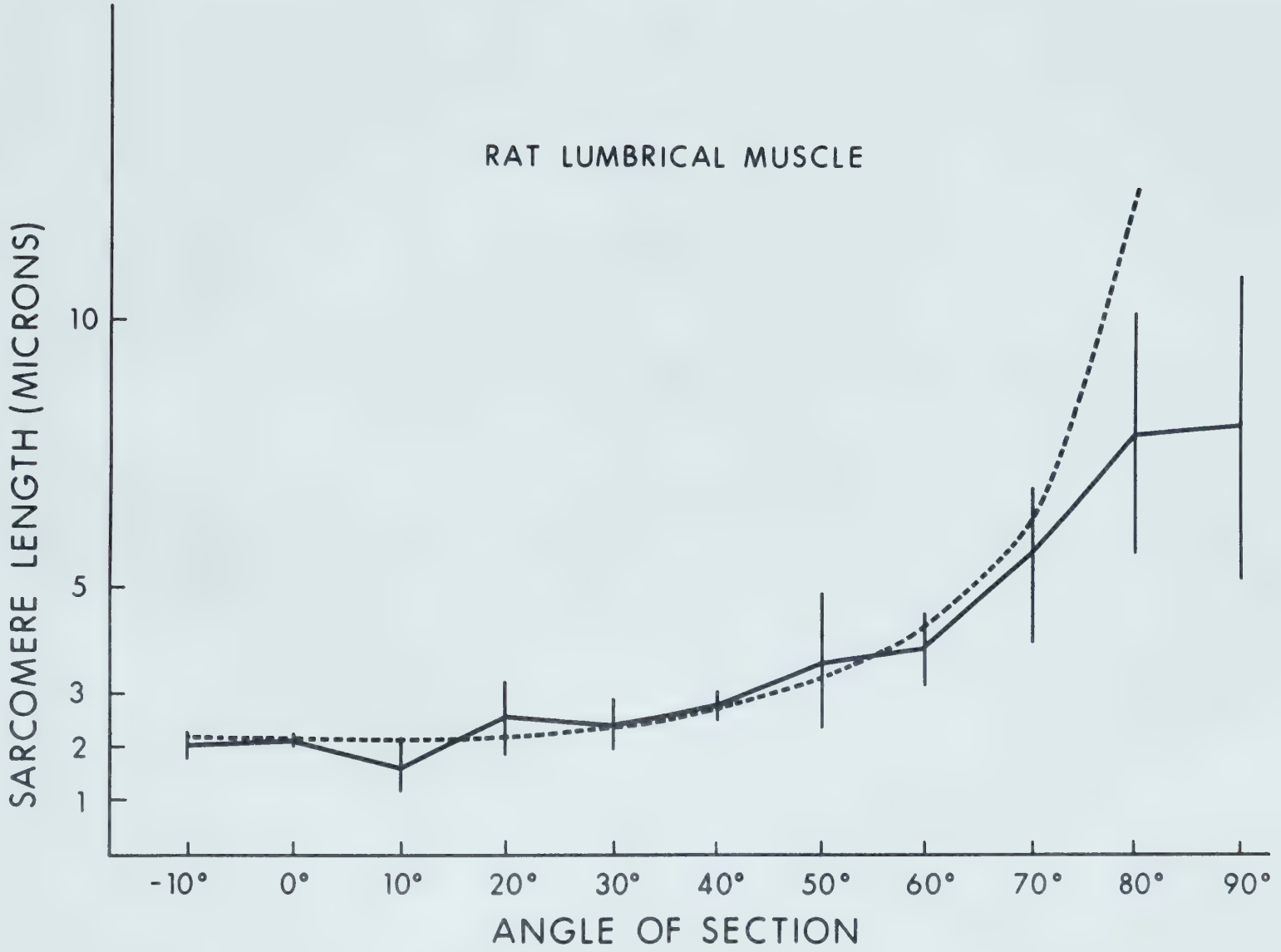
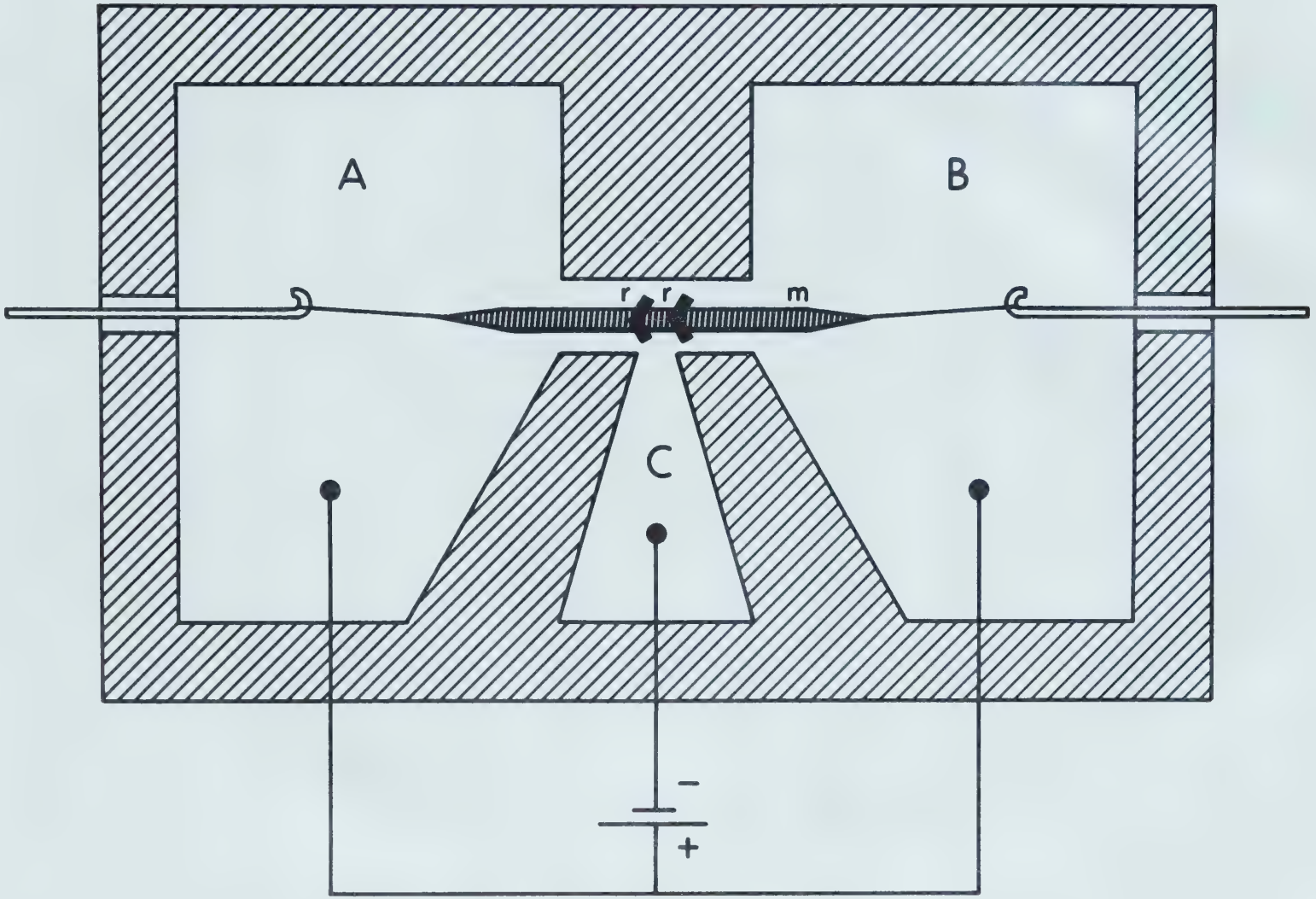

RAT LUMBRICAL MUSCLE
SARCOMERE LENGTH (MICRONS)
10
5
3
2
1
-10°
0°
10°
20°
30°
40°
50°
60°
70°
80°
90°
ANGLE OF SECTION

Fig. 11 Diagram of Apparatus for Local Stimulation of Sarcomeres in Small Bundles of Muscle Fibers

A diagram of an experiment to demonstrate local movement of the sarcolemma with local contraction of the sarcomere is shown. Hatched lines represent the perspex walls of a shallow chamber on a microscope slide. A small bundle of muscle fibers suspended between two hooks is indicated by m. Small silver riders are indicated by r. The chambers A, B, and C were filled with choline Ringer's solution to prevent action potential propagation. Direct current passed from chambers A and B to chamber C caused local contraction of sarcomere at the central part of the muscle fibers. Movement of the riders toward each other indicated local movement of the sarcolemma and inferred radial attachment of the contractile elements of the sarcomere to the sarcolemma.


A
B
r
r
m
C
−
+

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